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Karolinska Institutet, Stockholm, Sweden

# **GENE EXPRESSION PROFILING IN PERIODONTITIS**

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# GENE EXPRESSION PROFILING IN PERIODONTITIS

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*Have patience, all things are difficult before they become easy”*

Saadi Shirazi 1184-1292

*To my family, with love*



## ABSTRACT

The chronic inflammatory disease periodontitis is characterized by destruction of periodontal tissue, i.e. the tissues that surround and support the teeth. This complex disease is multifactorial, involving oral pathogens, an unfavorable host inflammatory response, environmental and genetic factors, as well as an altered gene expression contributing to disease pathology.

Despite extensive research of excellent quality, the genes involved in periodontitis remain uncharacterized and the mechanisms responsible for the destruction of periodontal tissue are poorly understood. Here, in attempt to gain further insight into and identify biomarkers for this disease, we have characterized global gene expression in periodontitis-affected gingival tissue and gingival fibroblasts.

Initially, we employed microarrays to examine gene expression in gingival fibroblasts, the predominant cell type in gingival connective tissue, after evoking an inflammatory response in these cells with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). This inflammatory mediator up-regulated the expression of a wide range of genes and, furthermore, activated several signaling pathways involved in immune and inflammatory responses, in particular the toll-like receptor (TLR) signaling pathway. We subsequently confirmed for the first time that TNF- $\alpha$  enhances the expression of TLR2 in gingival fibroblasts at both the mRNA and protein levels. In addition, we found that the c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF- $\kappa$ B) signal transduction pathways, as well as prostaglandin 15d-PGJ<sub>2</sub>, which has been proposed to be anti-inflammatory, are involved in this up-regulation.

Next, we examined the transcriptome, employing RNA-sequencing, in periodontitis-affected and healthy gingival tissue from patients with periodontitis. We demonstrated, as expected, that the degree of inflammation in periodontitis-affected gingival tissue is more pronounced than in corresponding healthy tissue from the same individual. Cluster analysis revealed that the clustering depended on the degree of inflammation, rather than the individual from whom the tissue was taken, indicating the existence of a characteristic gene expression profile for periodontitis. In addition, we identified two novel genes, interferon regulatory factor 4 (IRF4) and Chemokine (C-C motif) ligand 18 (CCL18) that were up-regulated in association with periodontitis.

In our third study, we utilized the RNA-sequencing approach to characterize gene expression in a large number of gingival biopsies both from patients with periodontitis and healthy control subjects. Among the several genes that were significantly up- or down-regulated in periodontitis, the two most differentially expressed were mucin 4 (MUC4) and matrix metalloproteinase 7 (MMP7). This investigation provides a comprehensive map of the gene expression associated with periodontitis and, suggests that MUC4 and MMP7 might be potentially valuable biomarkers and clinical therapeutic targets for periodontitis.

In conclusion, the information we present here provides new insights into the molecular mechanisms underlying the etiology and progression of this chronic inflammatory disease.

## LIST OF SCIENTIFIC PAPERS

- I. **Haleh Davanian**, Tove Båge, Johan Lindberg, Joakim Lundeberg, Hernan Q. Concha, Margaret Sällberg Chen, Tülay Yucel-Lindberg. Signaling pathways involved in the regulation of TNF $\alpha$ -induced toll-like receptor 2 expression in human gingival fibroblasts. *Cytokine* 2012 Mar; 57 (3): 406-416.
  
- II. **Haleh Davanian\***, Henrik Stranneheim\*, Tove Båge, Maria Lagervall, Leif Jansson, Joakim Lundeberg, Tülay Yucel-Lindberg. Gene expression profiles in paired gingival biopsies from periodontitis-affected and healthy tissues revealed by massively parallel sequencing. *PLoS One* 2012 Sep; 7(9): e46440. Doi: 10.1371/journal.pone.0046440.  
\*These authors contributed equally to this work.
  
- III. Anna Lundmark\*, **Haleh Davanian\***, Tove Båge, Gunnar Johannsen, Joakim Lundeberg, Tülay Yucel-Lindberg. Transcriptome sequencing in periodontitis. *Submitted Manuscript*.  
\*These authors contributed equally to this work.



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## LIST OF ABBREVIATIONS

15d-PGJ <sub>2</sub>	15-deoxy- $\Delta$ 12,14-prostaglandin J <sub>2</sub>
cDNA	Complementary DNA
CCL2	Chemokine (C-C motif) ligand 2
CD	Cluster of differentiation
cPLA <sub>2</sub>	Cytosolic phospholipase A <sub>2</sub>
Cy	Cyanine
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNase	Deoxyribonuclease
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCF	Gingival crevicular fluid
GO	Gene ontology
ICAM-1	Intercellular adhesion molecule-1
IL-1 $\beta$	Interleukin-1 $\beta$
IRAK1	Interleukin-1 receptor associated kinase 1
IRF3	Interferon regulatory factor 3
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MCP-1	Monocyte chemo attractant protein-1
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response protein 88
NF- $\kappa$ B	Nuclear factor-kappa B
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction

PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
<i>P.gingivalis</i>	<i>Phorphyromonas gingivalis</i>
PMN	Polymorphonuclear leukocyte
PPAR- $\gamma$	Peroxisome proliferator-activated receptor gamma
RNA-seq	RNA sequencing
Ro	Ro 106-9920
RT-PCR	Reverse transcriptase-PCR
SP	SP600125
STAT1	Signal transducers and activators of transcription 1
TIMPs	Tissue inhibitors of metalloproteinases
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
TLR	Toll-like receptor
qPCR	Quantitative PCR

# INTRODUCTION



**P**eriodontitis is a complex chronic inflammatory disease characterized by destruction of periodontal tissue. The disease involves numerous features, including altered gene expression contributing to disease pathology. Since no genes specifically related to this disease have yet been identified, we have employed here sequenced based analysis of the transcriptome to explore global gene expression in association with periodontitis, using gingival tissue and gingival fibroblasts.

## PERIODONTITIS

In 1700 researchers began to study two dental health problems they called gingivitis and periodontitis, collectively referred to as periodontal diseases and characterized by inflammation of the periodontium, i.e. the tissues that surround and support the teeth.<sup>1</sup> The periodontium is comprised of the cementum of the root surface, the alveolar bone, the periodontal ligament, and the gingiva,<sup>2-4</sup> divided anatomically into the unattached (marginal) gingiva at the terminal edge and with an outer surface, the papilla, and the attached gingiva extending apically from the marginal gingiva to eventually merge with the alveolar mucosa. Collagen fibers of the connective tissue firmly attach the epithelium of the gingiva to the underlying cementum and bone,<sup>5</sup> where it covers and protects the alveolar bone and surrounding teeth from environmental dangers such as microorganisms and harmful agents. This protection was historically thought to be provided simply by the physical barrier, but through the years the possibility that gingival epithelial cells play a role in innate immune responses to bacteria has been raised.<sup>5</sup>

In the early stage of periodontal disease, referred to as gingivitis, inflammation due to poor oral hygiene that result in deposit of bacteria as dental plaque is limited to the gingiva.<sup>6,7</sup> This condition is reversible, through improvement of dental hygiene.<sup>8</sup> However, even though the gingivitis may remain stable, if the dental plaque continues to irritate the gingiva, in some susceptible individuals the inflammation will become chronic and spread to deeper layers of the periodontium.<sup>9</sup> This process may in turn lead to destruction of the structures supporting the teeth, including the gingival tissue and underlying alveolar bone, a condition referred to as periodontitis.<sup>9</sup>

In the case of periodontitis, the periodontal ligament that attaches the gingiva and the alveolar bone to the cementum may detach during the inflammatory response to destructive stimuli such as lipopolysaccharide LPS, toxins and enzymes released by pathogens.<sup>10</sup> In susceptible individuals, inflammation can become chronic, leading to the formation of a pocket next to the tooth that exposes the cementum of the root surface and permits bacteria to accumulate, causing these same pockets to deepen and destroy underlying supporting tissues. If the disease becomes severe, teeth will loosen, become painful and eventually be lost.<sup>2,4,11</sup>

Although only approximately 5-20% suffer from severe periodontitis, the mild to moderate form can afflict most adults.<sup>12-15</sup> However, it must be emphasized that this prevalence

depends on the type of periodontitis, as well as the population involved.<sup>13,16</sup> The frequency increases with age<sup>17,18</sup> and men have been reported to be more susceptible than women.<sup>19</sup>

During the past four decades, the complex multifactorial nature of periodontitis has become obvious. Thus, accumulation of bacteria alone is no longer considered sufficient to initiate the disease, since individuals are not equally susceptible to and differ in their responses to bacterial infections. Oral pathogens, an unfavorable host inflammatory response, and other environmental and genetic factors are all now thought to be risk factors (Figure 1).<sup>2,20,21</sup> For example, several twin studies have revealed that inherited factors are involved in more than half of the cases of periodontal disease.<sup>22-24</sup> Furthermore, several investigations have shown that not only are smokers more likely to develop periodontitis than non-smokers,<sup>25,26</sup> but they also tend to develop more severe disease with more extensive tooth loss.<sup>25</sup> Moreover, smoking attenuates the response to periodontal treatment.<sup>27</sup> Recently, it was also suggested that oral pathogen communities, involved in periodontitis, do not only mediate inflammation at local sites but also affect systemic health.<sup>28</sup> Associations between periodontitis and systemic diseases such as diabetes mellitus,<sup>29,30</sup> rheumatoid arthritis,<sup>31-33</sup> cardiovascular diseases,<sup>34,35</sup> osteoporosis,<sup>36</sup> and obesity<sup>37</sup> have been proposed, suggesting that preventing and treating periodontitis may also improve these conditions.<sup>36,38</sup>

In summary, the key determinant of periodontitis is the complex interplay between microbial challenge and the subsequent host immune response, described below, in addition to modifying factors mentioned above.<sup>20,28</sup>

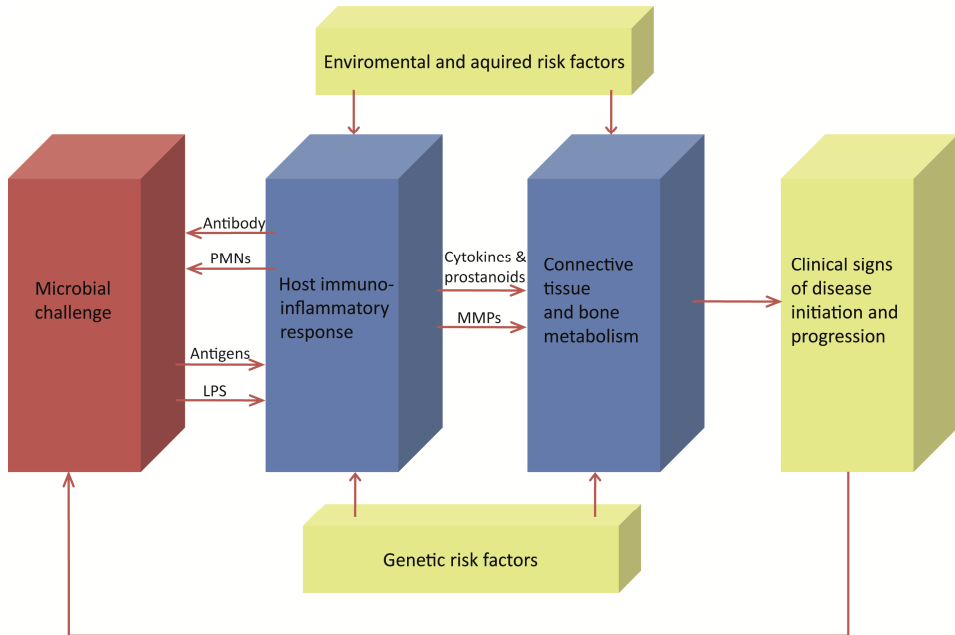
## Microbial challenge

Although bacterial plaque is required for initiation of gingivitis and periodontitis, the aerobic and anaerobic bacterial species present in the oral cavity, estimated to be more than 700 in number,<sup>6,39</sup> live essentially in symbiosis with the healthy host,<sup>2</sup> as they do on other external surfaces of the body and in the gut. Bacterial species adhere to and form highly organized biofilms on various oral sites, including the tooth surface and gingival margins. Each biofilm is a complex community containing a variety of microorganisms, facilitates the adhesion of other bacteria and produces an extracellular polysaccharide matrix that protects the microorganism community from the surrounding environment.<sup>40</sup>

During the development and progression of periodontitis, bacterial plaque accumulates deeper in the periodontal pocket, a more anaerobic environment, where the composition of the biofilm changes from primarily Gram-positive bacteria to more harmful anaerobic and Gram-negative bacteria, including the “red complex” pathogens, *Porphyromonas gingivalis* (*P.gingivalis*), *Tannerella forsythia* and *Treponema denticola*.<sup>41-43</sup> The red complex, the most virulent organisms present at diseased sites, is associated with more advanced periodontal disease.<sup>43</sup> These pathogens release tissue-degrading enzymes such as collagenases, fibrinolysins and other proteases, as well as other harmful bacterial products, including LPS, lipoteichoic acids and peptidoglycans collectively termed Pathogen-Associated Molecular



Patterns (PAMPs).<sup>44-46</sup> The presence of pathogens and their harmful products evokes an inflammatory response by the host designed to eliminate the microbial challenge.



**Figure 1. A schematic model of pathogenesis of periodontitis.** Periodontitis is a complex disease involving multifactorial causes, including microbial challenge, host immuno-inflammatory response and genetic fators, as well as enviromental and aquired factors. Adapted from Page 1997,<sup>1</sup>Abbreviations: PMNs, polymorphonuclear leukocytes; LPS, lipopolysaccharide; MMPs, matrix metalloproteinases.

## Host immune response

### Toll-like receptors

The first step in the host immune response, i.e., recognition of PAMPs, expressed on or released by invading pathogens,<sup>47,48</sup> is accomplished by toll-like receptors (TLRs) on the plasma membrane. To date, 10 and 12 functional TLRs that recognize specific ligands have been identified in humans and mice, respectively.<sup>48-50</sup> Upon ligand binding these receptors initiate a cascade of signaling pathways leading to the production and release of chemokines and adhesion molecules that lead to migration of neutrophils and production of mediators that initiate an inflammatory response.<sup>51,52</sup>

Two types of signaling pathways are involved, one dependent on and the other independent of the myeloid differentiation primary response protein 88 (MyD88). While most TLRs act via the dependent pathway, stimulation of TLR3 and TLR4 instead activates interferon-regulatory factor 3 (IRF3).<sup>53,54</sup> Upon ligand binding to TLRs, MyD88 recruits interleukin-1 receptor associated kinase 1 (IRAK1) to induce the mitogen-activated protein kinase kinase

kinase (MAP3K), which in turn activates the mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinases (JNK) and p38, as well as the transcription factor nuclear factor-kappa B (NF- $\kappa$ B). This process results in the inflammatory response designed for pathogen clearance.

TLRs are expressed predominantly on the surface of cells of the innate immune system, including neutrophils, monocytes/macrophages and dendritic cells. However, cells of the periodontium, including epithelial cells and gingival fibroblasts also express these receptors and thereby participate in the innate immune response against bacteria that cause dental plaque.<sup>51</sup> It has been proposed that gingival epithelial cells express TLRs, as a result of constantly being exposed to bacterial PAMPs.<sup>51,55</sup> In addition, gingival fibroblasts express TLR2 and TLR4 upon exposure to LPS from *P.gingivalis*.<sup>56,57</sup>

### **The inflammatory response**

Recognition of PAMPs by TLRs on inflammatory and resident cells of the gingiva, including fibroblasts, evokes an immediate inflammatory response involving the production of cytokines and chemokines that recruit appropriate cells to the site of infection.<sup>7,20,58</sup> The subsequent cascade of events is designed to meet the microbial challenge. Vasoactive amines and preformed tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are released by mast cells, thereby increasing vascular permeability so that components of the blood can enter the connective tissue. These mediators also up-regulate expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells.<sup>7,44</sup>

The enhanced vascular permeability allows polymorphonuclear leukocytes (PMNs) to enter the tissue, where they release lysosomal enzymes that degrade connective tissue.<sup>7</sup> By this time, lymphocytes (mainly T-cells) and macrophages have also begun to invade the tissue and release pro-inflammatory mediators, including interleukin-1 $\beta$  (IL-1 $\beta$ ), TNF- $\alpha$ , and prostaglandins, as well as hydrolytic enzymes such as matrix metalloproteinases (MMPs). Together, these processes degrade 60-70% of the collagen of the connective tissue at the affected site, but this does not necessarily result in permanent damage, since the gingival tissue can repair and remodel with the alveolar bone still intact.<sup>4,7</sup> However, if the inflammation fails to resolve and becomes chronic, with a B-cell/plasma cell response, additional connective tissue and even alveolar bone can be lost.<sup>7</sup> Thus, although this initial immune response to a microbial challenge in periodontal tissue is a physiological defense mechanism, in certain individuals with greater susceptibility and/or exposure to unfavorable environmental factors, the inflammation becomes chronic and pathological.

### **Inflammatory mediators**

Even though the release and production of pro-inflammatory mediators in periodontitis is initially protective, the majority of the tissue damage that characterizes the clinical signs of the disease is derived from the prolonged and excessive production of various mediators.

These mediators include cytokines and chemokines, proteolytic enzymes (MMPs), as well as prostaglandins, which all play a major role in the pathogenesis of periodontitis.<sup>45</sup>

Cytokines play essential roles in several physiological processes, but can also promote pathological processes if the balance between pro- and anti-inflammatory cytokines tips towards pro-inflammatory, as occurs in periodontitis. In the periodontium, cytokines are produced by invading inflammatory cells (neutrophils, macrophages and lymphocytes), as well as by resident cells of the gingiva (epithelial cells and fibroblasts).<sup>45,59</sup> The pro-inflammatory cytokines implicated in the pathogenesis of periodontitis include IL-1, IL-6, IL-12, IL-17, IL-18, IL-21, TNF- $\alpha$  and interferon gamma, with IL-1 $\beta$  and TNF- $\alpha$  appearing to play pivotal roles.<sup>45,59,60</sup>

TNF- $\alpha$  is crucial for the initiation of an inflammatory response and, ultimately, tissue destruction.<sup>59,61</sup> This cytokine is produced and secreted at a very early stage by mast cells, but as the inflammatory process progresses, several other types of cells, including PMNs, macrophages, epithelial cells, fibroblasts, endothelial cells and osteoclasts also start to produce and secrete TNF- $\alpha$ .<sup>45,59</sup> TNF- $\alpha$  promotes the expression of adhesion molecules, chemokines and MMPs, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production, and osteoclast formation.<sup>59</sup> The levels of this pro-inflammatory mediator in gingival crevicular fluid (GCF) and gingival tissue at sites of periodontitis is elevated<sup>62-64</sup> and, moreover, anti- TNF- $\alpha$ -treatment has been reported to reduce loss of tooth attachment in patients with periodontitis and subsequent rheumatoid arthritis.<sup>65</sup>

Chemokines are chemotactic cytokines produced by a variety of cells, including endothelial, epithelial and stromal cells, fibroblasts, as well as leukocytes.<sup>66</sup> In connection with the inflammatory response, chemokines promote migration of phagocytic cells to the site of infection, as well as other relevant processes such as angiogenesis, cell proliferation, and apoptosis.<sup>67,68</sup> The two key chemokines of the innate immune system are IL-8 and monocyte chemoattractant protein-1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2).<sup>69</sup> Synthesized by gingival fibroblasts, gingival epithelial cells, as well as endothelial cells in the periodontium in response to IL-1, TNF- $\alpha$  and LPS, IL-8 acts as a chemoattractant for PMN leukocytes.<sup>70-72</sup> The level of this chemokine in gingival tissue at sites of periodontitis, characterized by large numbers of PMN cells, is elevated.<sup>73</sup> MCP-1 initiates and regulates mobilization of monocytes to active sites of severe periodontal inflammation,<sup>69</sup> and its expression in the gingival tissue of patients with periodontitis is enhanced.<sup>74,75</sup> Furthermore, the level of MCP-1 in GCF from patients with chronic periodontitis is elevated.<sup>76</sup>

The large family of MMP endopeptidases degrades extracellular matrix molecules such as collagen and proteoglycans and thereby participates in tissue remodeling. These enzymes are expressed by several cells of the periodontium, including fibroblasts, keratinocytes, osteoblasts, endothelial cells, macrophages, and neutrophils.<sup>60,77,78</sup> In healthy gingival tissue, collagen homeostasis is maintained by endogenous tissue inhibitors of metalloproteinases (TIMPs),<sup>77,78</sup> which regulate the expression of MMPs, primarily by

fibroblasts.<sup>79</sup> In inflamed periodontal tissue, several resident cells and inflammatory cells (particularly neutrophils) secrete excessive levels of MMPs, thereby disrupting the balance and resulting in degradation of connective tissue. Several MMPs have been shown to be associated with periodontitis, in particular MMP2, MMP8 and MMP9.<sup>80,81</sup> For example, the levels of MMP8 and MMP9 in GCF and gingival tissue are reported to be directly correlated to the severity of disease.<sup>82-84</sup>

Prostaglandins, additional important mediators of host inflammatory responses, are synthesized from the long-chain arachidonic acids by inflammatory cells, as well as by fibroblasts and other cells resident in the gingival tissue. Among prostaglandins, PGE<sub>2</sub> plays a major role in the pathogenesis of periodontitis,<sup>85,86</sup> being present at elevated levels in the gingival tissue and GCF of patients with this disease. PGE<sub>2</sub> induces secretion of MMPs and stimulates formation of osteoclasts through the receptor activator of NF-κB ligand (RANKL), which is also associated with periodontal tissue destruction.<sup>85,87</sup>

## GENE EXPRESSION

In 1956 Francis Crick formulated the Central dogma of molecular biology, describing the flow of genetic information from DNA to a gene product. This process occurs in two steps: DNA is first transcribed into messenger RNA (mRNA), which then serves as a template for protein synthesis during translation. Whereas the genetic information, the genome, is the same in every cell of a given organism (with the exception of gametes), the mRNA and protein profiles, i.e., the transcriptome and proteome, vary between cells.

The term *gene expression* refers to the process of transferring the information from a gene to mRNA and then decoding this information to make a protein. The patterns of gene expression in a cell or tissue depend on its developmental stage, function or disease state and determination of the profile of the mRNA molecules present can provide insights into the mechanisms underlying pathological processes. For example, gene expression profiling provides a tool for answering a wide range of questions, including how active various genes are in different types of cells or tissues, how gene expression levels change under different conditions, and how genes are “*differentially expressed*” between diseased and healthy tissues. In the following sections of this thesis, gene expression will be monitored at the mRNA level.

## Techniques for studying gene expression

Over the years, analysis of gene expression has undergone major advances, from the first northern blot to the development of the polymerase chain reaction (PCR) and high-throughput procedures, including microarrays and massive parallel sequencing, leading researchers to investigate gene expression at a level never possible before. The choice of approach depends intimately on the questions being asked. In biological systems where the genes of interest are not yet known, primary characterization is usually performed

employing high-throughput technology. However, for investigating many samples where the key genes of interest have been identified, more targeted techniques, such as PCR, are commonly used.

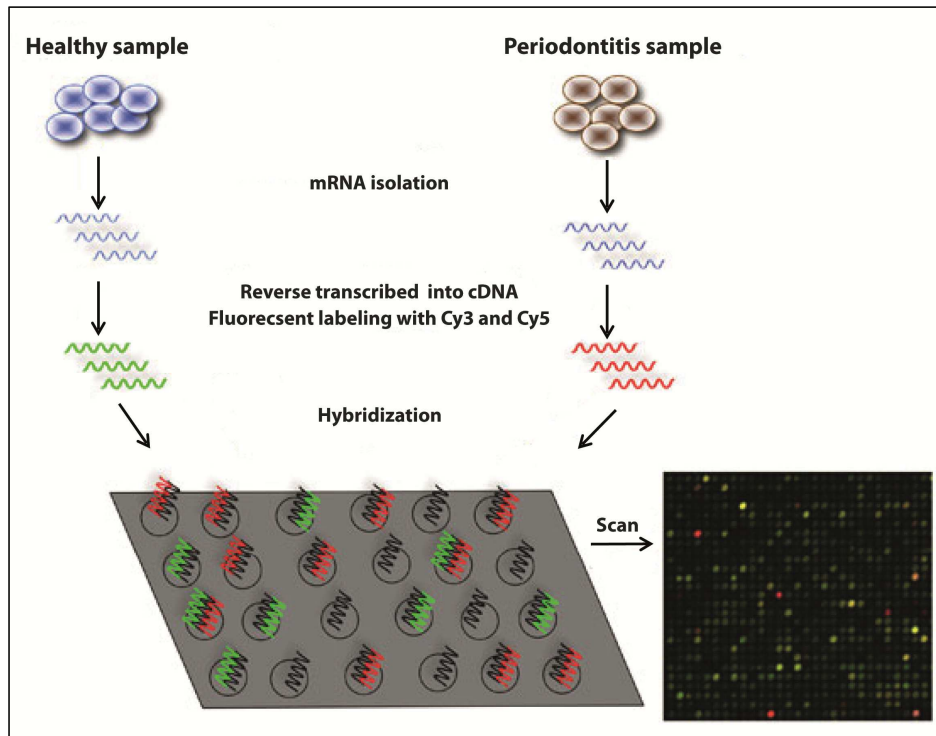
PCR, the cornerstone of the human genome project, was developed by Kary Mullis almost 30 years ago, winning him the Nobel Prize for chemistry in 1993.<sup>88-90</sup> PCR technology is best suitable for measuring the expression of few genes in a large number of samples.<sup>91</sup> Reverse transcriptase-PCR (RT-PCR) provides qualitative gene expression profiling through the construction of cDNA transcripts from RNA during the final phase of the reaction. In contrast quantitative real-time-PCR or qPCR, the most sensitive technique for quantifying mRNA levels and often considered the gold standard,<sup>91</sup> utilizes fluorescent probes to quantify the amplification of DNA during the early phases of the reaction. Among the distinct advantages of qPCR,<sup>92,93</sup> is its more extensive dynamic range of detection (capable of revealing as little as 2-fold change), in contrast to the 10-fold changes detectable on the agarose gels used in RT-PCR. Furthermore, qPCR data are expressed directly as numbers, eliminating the post-processing required with RT-PCR.<sup>92</sup> The PCR technology has been widely used for research purposes, as well as for detection of pathogens, and diagnosis of genetic disorders.

In the present work we have applied both targeted techniques, such as RT-PCR, and high-throughput methodology, including microarrays and RNA sequencing (RNA-seq) (see further below).

## **Microarrays**

In 1995 Patrick Brown and colleagues at Stanford University first brought attention to the exciting potential of using microarray technology to determine the expression of numerous genes at the same time.<sup>94-96</sup> A microarray is a glass or silicon slide or chip on which are immobilized 500-30 000 complementary DNA (cDNA) fragments (usually as long as 1500 bp) or oligonucleotides (usually 70-mers), often several for each gene being analyzed.<sup>97</sup> Typically, mRNA is first isolated from two samples to be compared (e.g. healthy and periodontitis sample from the same individual) and subsequently reverse transcribed by RT-PCR to generate cDNA. The two samples of cDNA are then labeled individually with different fluorescent dyes e.g. Cyanine (Cy; Cy3 and Cy5), pooled and cohybridized onto the arrays, from which a dual colour image is finally obtained by scanning (Figure 2).<sup>94</sup> Since microarray analysis provides only the relative levels of different mRNAs, Northern blot and qPCR are commonly applied to confirm the data obtained.

The microarray approach is a rather simple, inexpensive but robust means of examining the expression of thousands of genes at once and we employed this technique in Study I to characterize the mRNA profile of human gingival fibroblasts with and without exposure to TNF- $\alpha$  this choice was based on our previous experience with microarrays, as well as the considerable collective experience concerning normalization and interpretation of the data thus obtained. The deep-sequencing technology, discussed below, was at that time relatively new and required extensive validation and advanced tools for data analysis.



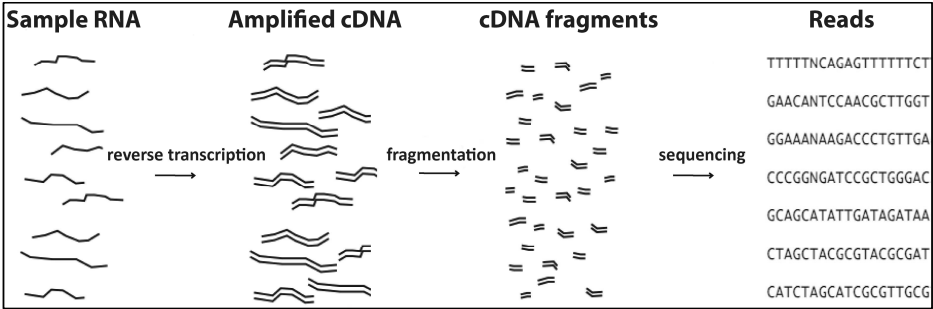
**Figure 2. Schematic illustration of a typical microarray analysis.** mRNA is isolated and purified from samples and reverse transcribed to cDNA. The cDNAs are fluorescently labeled with Cy3 and Cy5, and thereafter pooled and cohybridized onto the microarray. Afterwards dual colour images are generated upon scanning the microarray. Abbreviation: Cy, cyanine.

## Massive parallel sequencing, RNA sequencing

In 2009, a novel deep-sequencing technology, termed RNA-sequencing (RNA-seq) revolutionized transcriptome analysis. Unlike microarray technology, RNA-seq does not require labeling with dyes or designing probes with a pre-defined set of sequences,<sup>98,99</sup> thus, allowing global characterization of the transcriptome.<sup>97,98</sup> Of the several systems presently available for RNA-seq including Illumina IG18,<sup>100-102</sup> Applied Biosystems SOLiD<sup>103</sup> and Roche 454 Life Science,<sup>104-106</sup> the Illumina sequencer is most commonly used.<sup>107</sup>

RNA-seq has considerable potential to eventually replace microarrays for gene expression profiling. In addition to its independence from knowledge of reference transcriptomes, RNA-seq is more reproducible, involves lower background signals and less technical variation, and covers a wider range of expression levels.<sup>108,109</sup> Moreover, this approach can identify novel gene transcripts, novel isoforms, allele-specific expression and alternative splicing variants.<sup>110,111</sup> However, many research groups still use microarrays, which are cheaper, and do not require the extensive bioinformatics skills and computer resources needed for the retrieval, storage and processing of the large amounts of data provided by RNA-seq.<sup>98,112</sup>

In practice, RNA is isolated from a sample and purified; converted to a library of cDNA and fragmented; and adaptors attached to these fragments, which are then amplified and sequenced for short distances from one (single-end sequencing), or both ends (pair-end sequencing) (Figure 3).<sup>112</sup> The millions of short reads generated can be mapped to a reference genome and the number of reads that align to each individual gene, referred to as “counts”, provides a digital measurement of the level of gene expression.<sup>113</sup>



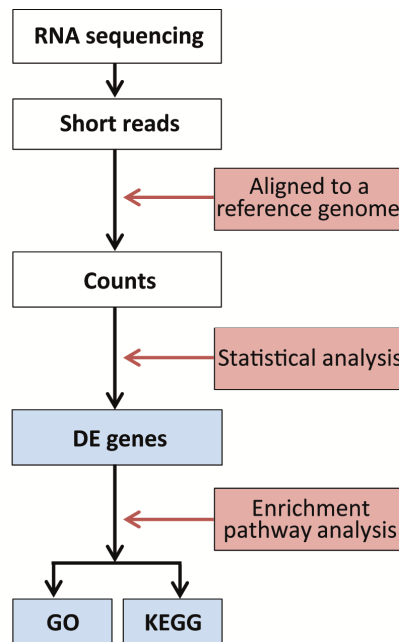
**Figure 3. Flowchart of a RNA sequencing experiment.** RNA is isolated from samples and purified, reverse transcribed to cDNA and thereafter fragmented. These cDNA fragments are sequenced and millions of reads generated.

### Analysis of sequencing data

Once reads are generated through RNA-seq, gene regions that match read sequences are identified and the total number of reads that map onto each gene provides an estimate of the level of gene expression (counts). Normalization is then performed to eliminate possible biases and allow reliable comparison between and within samples, which is absolutely necessary for differential gene expression analysis.<sup>112-114</sup> Differential gene expression analysis allows identification of genes whose levels of expression differ in two samples, and thus might play a potential role in, e.g. the pathology of a disease.<sup>113</sup> A 2-fold difference is usually considered to be a reasonable cutoff.<sup>113,115</sup>

The next step in the data analysis is to perform enrichment analysis. Enrichment analysis helps to interpret the mass of transcriptome profiling data generated by array and sequencing analysis, facilitating the discovery of possible associations and interactions of differentially expressed genes.<sup>116</sup> With this approach genes are mapped, e.g. to their related biological categories in Gene ontology (GO)<sup>117</sup> or components of pathways in Kyoto Encyclopedia of Genes and Genomes (KEGG),<sup>118</sup> and compared with the background distribution for statistical over- or under-representation.<sup>119</sup> By searching different databases, the GO project attempts to categorize all known genes according to their involvement in biological process, molecular functions, and cellular localization.<sup>117</sup>

Thus, enrichment analysis helps answer biological questions such as: is a disease phenotype characterized by a specific profile of differentially expressed genes? Which cellular/molecular pathways might be involved in the pathological process? Can any single gene or set of genes predict severity and/or outcome? And in the long run, can these findings be applied in clinical practice? A schematic overview of the steps included in the RNA-seq data analysis process is illustrated in Figure 4.



**Figure 4. Schematic overview of RNA sequencing data analysis.** The obtained reads from the sequencing are aligned to a reference genome and the counts are analyzed statistically. Then, enrichment pathway analysis of the differentially expressed (DE) genes is performed and these are mapped to e.g. GO categories and KEGG pathways. Abbreviations: Differentially expressed, DE; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

## Microarray analysis in periodontitis- previous studies

### In gingival tissues

Several research groups have investigated the gene expression profile in patients with periodontitis.<sup>120-124</sup> For example, in 2008 Demmer and co-workers<sup>121</sup> carried out a microarray-based whole tissue transcriptome study that demonstrated significant changes in the gene expression of genes related to 61 pathways, including apoptosis, antimicrobial humoral response, antigen presentation, regulation of metabolic processes, signal transduction, and angiogenesis, in papillae from diseased and healthy sites from patients with advanced periodontitis.<sup>121</sup> The same group attempted in 2013 to find a molecular difference between chronic and aggressive periodontitis based on the gingival transcriptome. However,



their microarray study could not differentiate between the two forms of periodontitis,<sup>123</sup> but in 2014 on the basis of these same microarray data they proposed that distinct transcriptome profiles in pathologic gingival tissues reflect phenotypic differences and might provide a basis for a novel classification of the disease.<sup>124</sup>

In 2008 Beikler and colleagues<sup>125</sup> used microarrays to examine a selected number of inflammatory genes and found that following therapy of periodontal tissues the activities of biological pathways responsible for tissue damage and regulation of repair were higher, while the expression of immune and inflammatory genes was lower than in healthy controls.<sup>125</sup> However, in light of individual variations in gene expression and the fact that the control tissues were not from the same patients, their study population of 11-12 individuals was small and the results should therefore be interpreted with caution.

### **In gingival cells**

*In vitro* efforts have been made to characterize the gene expression profiles of different cell populations from the gingiva. In 2003, Wang et al.<sup>126</sup> employed microarray analysis, to demonstrate that eight different genes, including the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ , were more highly expressed in fibroblasts from inflamed than healthy gingiva.<sup>126</sup> To further evaluate the role of the pro-inflammatory cytokine TNF- $\alpha$  in this context, Båge and colleagues<sup>127</sup> utilized microarray analysis to reveal that the signal transduction pathways NF- $\kappa$ B and JNK are positively regulated by TNF- $\alpha$  in gingival fibroblasts and that inhibition of these pathways attenuates the expression of prostaglandins. These researchers concluded that these two pathways are potential targets for inhibiting the chronic inflammation involved in periodontitis.<sup>127</sup>

Using an epithelia-specific cDNA microarray to analyze gene expression in human gingival keratinocytes stimulated with IL-1 $\beta$  *in vitro*, Steinberg and co-workers<sup>128</sup> showed that several genes involved in pathways such as cell stress, DNA repair, cell cycle and proliferation, anti-pathogen response, extracellular matrix turnover, and angiogenesis were differentially expressed.<sup>128</sup> Furthermore, when Papapanou and co-workers,<sup>129</sup> characterized mRNA levels in peripheral blood isolated from patients being treated for periodontitis, approximately 1/3 exhibited substantial increases in the expression of genes associated with innate immunity, apoptosis and cell signaling. The authors concluded that periodontal therapy might involve anti-inflammatory changes.<sup>129</sup>

In conclusion, despite much research of excellent quality, the broad spectrum of genes involved in periodontitis remains uncharacterized and the mechanisms responsible for the destruction of periodontal tissue are still poorly understood. Here we focus on determining the transcriptome of periodontitis utilizing RNA-seq technology, in attempt to increase the knowledge of underlying mechanisms and identify putative biomarkers for the disease.



## **AIMS OF THE PRESENT THESIS**



**T**he general aim of this thesis was to identify genes involved in periodontitis, through whole transcriptome analysis of gingival tissue and gingival fibroblasts. Revealing its global gene expression profile and interactions between associated genes can better our understanding of the pathogenesis of this complex chronic inflammatory disease.

## **STUDY I**

Study I was designed to characterize the effects of the pro-inflammatory cytokine TNF- $\alpha$  on the expression and regulation of TLR2 in human gingival fibroblasts. Subsequently, microarrays were used to explore the involvement of various signal transduction pathways in this context.

## **STUDY II**

Study II investigated the gene expression profile of diseased and healthy gingival tissues from patients with periodontitis, utilizing RNA-seq.

## **STUDY III**

The aim of Study III was to map the gene expression pattern in gingival tissue from a large cohort consisting of both patients with periodontitis and healthy controls. Furthermore, we confirmed that the differentially expressed mRNAs reflected changes at the protein level as well, thereby identifying potential biomarkers and therapeutic targets for periodontitis.

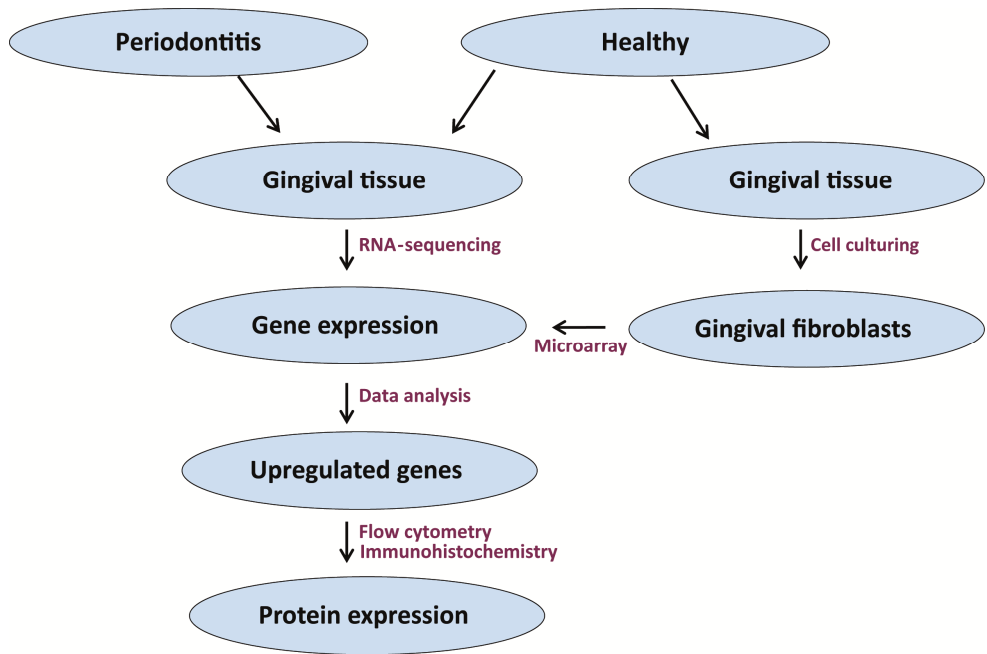


## **MATERIALS AND METHODS**





This section presents an overview of the study design, recruitment of the study population and techniques employed with special emphasize on gene expression profiling. For more detailed protocols, the reader is referred to the Materials and Methods sections of Studies I-III.



**Figure 5. Schematic overview of the study design.** The gene expression profile of human gingival fibroblasts was investigated by microarray analysis. RNA sequencing technology was utilized to characterize gene expression in gingival tissue biopsies both from patients with periodontitis and from healthy control subjects. The genes up-regulated in cells and gingival tissues, was examined further at the protein level by flow cytometry and immunohistochemical analysis.

## ETHICAL CONSIDERATIONS

All the studies were performed in accordance with the principles for medical research as formulated in the Declaration of Helsinki and current legislation in Sweden. Collection of the gingival biopsies was pre-approved by the Regional Ethics Board in Stockholm. Written informed consent was obtained from the patients with periodontitis, as well as from the healthy control subjects, prior to enrollment in the studies.

## STUDY DESIGN

The design of our project, the main purpose of which was to examine gene expression pattern in connection with periodontitis, is illustrated schematically in Figure 5. The project began with the establishment of an *in vitro* model using gingival fibroblasts from periodontally healthy subjects, in which the gene expression profile was characterized utilizing microarray technology in Study I. In the next two studies (II and III), we used RNA-seq to investigate gene expression in gingival tissue biopsies both from patients with periodontitis, as well as from healthy control subjects. Genes that were up-regulated in cells and gingival tissue biopsies were examined further at the protein level, by flow cytometry and immunohistochemical analysis.

## GINGIVAL FIBROBLASTS (STUDY I)

Study I involved cultures of human gingival fibroblasts established from biopsies obtained from 5 healthy individuals 6-12 years of age with no clinical signs of periodontal disease. Minced gingival tissue was explanted to tissue culture flasks containing culture medium supplemented with penicillin and by trypsinization gingival fibroblasts were obtained and grown at 37°C under 5% CO<sub>2</sub> and subjected to 4-10 passages before use. The cells were seeded onto Petri dishes in culture medium supplemented with 5% fetal calf serum, cultured for 24 h at 37° C, and then exposed to the pro-inflammatory cytokine TNF- $\alpha$  in the absence or presence of various effectors of intracellular signaling pathways. Control cells remained in culture medium alone. After incubation for different periods, the cell monolayer was rinsed and the cells collected for microarray, RT-PCR or flow cytometric analysis.

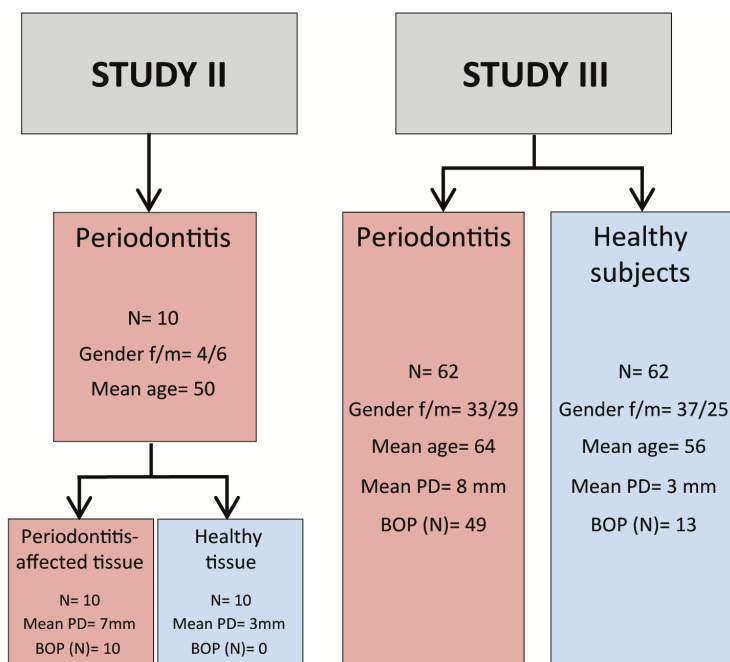
## GINGIVAL TISSUE BIOPSIES (STUDIES II AND III)

### The study population

Gingival bleeding, radiographic assessment of bone loss, clinical attachment level, and probing depth have traditionally been the basic clinical criteria for diagnosis of periodontitis.<sup>130</sup> Using these criteria, 10 patients diagnosed with periodontitis were recruited for Study II and 62 patients diagnosed with periodontitis for Study III (Figure 6). All demonstrated at least one tooth site with a probing depth  $\geq$  6 mm, a clinical attachment level

$\geq 5$  mm, as well as bleeding upon probing. None of these patients used nicotine or nicotine-replacement medications.

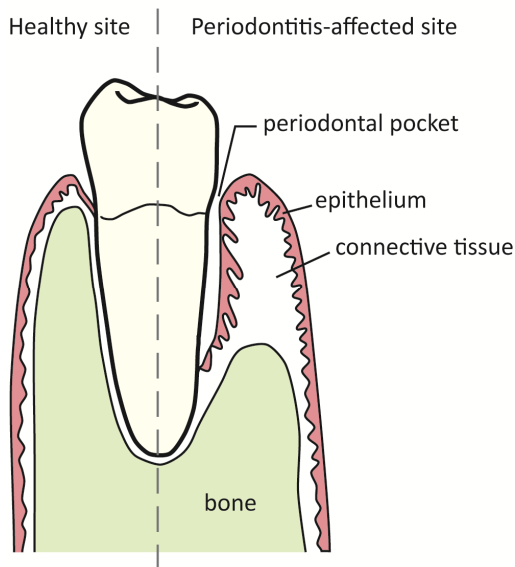
A total of 62 subjects were also included in the healthy control group for Study III (Figure 6). These individuals were recruited in connection with implant surgery for tooth loss due to reasons other than periodontitis (e.g. caries, accidents, etc.), which gave us the possibility to collect biopsies at the same time. The inclusion criteria were lack of any signs of periodontal disease, i.e. no sites with gingival/periodontal inflammation, a probing depth  $\leq 3.0$  mm, a clinical attachment level  $\leq 3.5$  mm and no bleeding upon probing. None of these subjects used nicotine or nicotine-replacement medications either.



**Figure 6. Overview of the study populations in Studies II and III.** 10 patients diagnosed with periodontitis (mean age=50) were recruited for Study II, periodontitis-affected and healthy gingival biopsies were collected from all 10. In Study III, 62 diagnosed with periodontitis (mean age=64) and 62 healthy controls (mean age=56) were included and gingival biopsies collected from each. Abbreviations: N, numbers; PD, probing depth; BOP, bleeding on probing; f/m, female/male.

## Collection of biopsies

In Study II both periodontitis-affected tissue and healthy tissue were collected from each individual. In connection with flap surgery, two gingival biopsies were collected from a periodontal pocket affected by periodontitis, and two other biopsies of about the same size (approximately 2x2 including the connective tissue and epithelium) from a clinically healthy gingival pocket (i.e. no gingival/periodontal inflammation, no bleeding upon probing, a probing depth  $\leq 3.5$  mm and a clinical attachment level  $\leq 3.5$  mm) (Figure 7). One biopsy from each site was stored in RNA Later for gene expression analysis and the other subjected to histological and immunohistochemical analysis.



**Figure 7. Schematic illustration of a healthy and a periodontitis-affected site in a patient diagnosed with periodontitis.**

Next, in Study III we examined the gene expression profile in 62 patients with periodontitis and 62 healthy individuals. Of the two gingival biopsies of about the same size collected at the same site in each participant, one was stored in RNA Later and the second fixed in formalin and embedded in paraffin.

## **Histological staining of biopsies**

To assess the degree of inflammation, the quality, as well as the orientation of the epithelium and connective tissue of the gingival biopsies in Studies II and III, traditional histological staining with hematoxylin and eosin (H&E) was performed. The deep blue-purple hematoxylin stains nucleic acids, while pink eosin stains proteins non-specifically. Accordingly, in a typical tissue, nuclei are stained blue, while the cytoplasm and extracellular matrix exhibit varying degrees of pink staining. The degree of inflammation in all gingival biopsies was scored on a relative scale from 0-3 by three “blinded” observers. Furthermore, infiltration as by T-cells, B cells and macrophages was determined more specifically using traditional immunohistochemical techniques involving commercial antibodies directed against cluster of differentiation (CD) 3, CD20 and CD68, respectively.

## **GENE EXPRESSION ANALYSES (STUDIES I-III)**

### **Reverse transcription-PCR (RT-PCR)**

In Study I RT-PCR was applied to investigate the expression of TLR2 in TNF- $\alpha$ -stimulated gingival fibroblasts. After 6 h of treatment, RNA was isolated and subjected to reverse transcription (Superscript II, 1.0  $\mu$ g total RNA in a total reaction volume of 20  $\mu$ l). TLR2 cDNA was amplified by PCR (5.0  $\mu$ l cDNA, AmpliTaq Gold DNA polymerase, final reaction volume of 50  $\mu$ l), as were the control GAPDH cDNA and negative controls without cDNA. Thereafter, 10  $\mu$ l of each PCR reaction product was run on an agarose gel containing a safe fluorescent nucleic acid dye (GelRed) together with a DNA-ladder standard and the intensity of the bands obtained quantified using the Chemidoc XRS molecular system.

### **Microarray analysis**

To gain more global insight into the gene expression profile of TNF- $\alpha$ -stimulated fibroblasts, microarray analysis was also performed in Study I. Fibroblasts from three different patients were analyzed at four different time-points. The microarray chip employed, printed at the KTH core facility, contained 35,344 70-mer probes representing 28,948 Entrez Gene IDs,<sup>131</sup> of which 17,972 are unique. RNA was isolated from cultured gingival fibroblasts treated with the pro-inflammatory mediator TNF- $\alpha$  for 1, 3 or 6 h utilizing the commercially available RNeasy Mini Kit and then quantified and tested for purity spectrophotometrically at 260/280 nm. cDNA was synthesized using Superscript III and random hexamer primers and then labeled with the fluorophore Cy3 (control cells, green) or Cy5 (TNF- $\alpha$ -stimulated cells, red). After confirming successful labeling and determining the fluorophore concentrations with a Nanodrop instrument, these concentrations were used to obtain equal amounts of sample.

The microarray chips were pre-hybridized with a buffer containing bovine serum albumin, the Cy3- and Cy5-labeled samples pooled and hybridization buffer added. The arrays were hybridized overnight at 42° C in a water bath and after washing, immediately scanned on a

Agilent G2565BA scanner. The GenePix 5.1.0.0 software was used to extract the raw signals from the TIFF images and to assign each spot an ID. The data were analyzed with different packages in the R software.<sup>132</sup> For low-resolution analysis, the raw data from GenePix was transferred into the R software, the spots filtered for removal of abnormal physical properties, and the slides subjected to print-tip Lowess normalization. For estimation of the M-value (i.e.  $\log_2$  of the fold-change, e.g. the level of mRNAs in TNF- $\alpha$ -treated cells/untreated cells) and variance for each gene, a linear model in the Bioconductor package<sup>133</sup> LIMMA (Linear models for microarray data) was established. Subsequently, differentially expressed genes were identified utilizing a moderated t-test,<sup>134,135</sup> with application of a false discovery rate algorithm to the calculated p-values to correct for multiple testing.<sup>136</sup> Thereafter, differentially expressed genes were defined as those with a q value < 0.05 (the false discovery rate analog of a p-value) so that the proportion of false positives among these genes was 0.05.

## **RNA sequencing (RNA-seq) analysis**

In Studies II and III the gene expression profile of gingival biopsies was characterized by deep high-throughput sequencing using the Illumina HiSeq 2000 sequencer. The biopsies were homogenized and lysed, to enable isolation of stable RNA and this RNA was then purified and treated with DNase to degrade contaminating DNA. The quality of the RNA thus obtained was assessed using the Agilent Bioanalyzer system. 2-3  $\mu$ g tissue with an RNA integrity number (RIN) >7 was used for the preparation of RNA employing reagents either from the Illumina mRNA Sample Preparation Kit or from vendors specified in the mRNA sample preparation protocol. The clustering of the bar-coded samples was performed on a cBot Cluster Generation System using an Illumina HiSeq Single Read Cluster Generation Kit. The library preparations were sequenced on an Illumina HiSeq 2000 as single-reads to 100 bp. Two sequencing runs were performed, where two and three lanes were used in the first and second sequencing runs, respectively.

In Study II, these runs generated 402 million reads (an average of 15 million reads per sample) that passed through the Illumina Chastity filter, while in Study III 40.2 million paired-end reads were obtained per sample. All sequences obtained were aligned to the human genome reference hg19 with TopHat version 1.1.4 and Samtools version 0.1.8 and annotations from Ensembl and RefSeq applied to assign features to genomic positions and these features counted using HTSeq version 0.4.6 with parameters -m intersection-strict -s no -t exon. The R/Bioconductor DESeq package was employed to identify differential gene expressions among the read counts generated by HTSeq and to perform hierarchical clustering of the samples. All replicates for the periodontitis-affected and healthy biopsies had  $R^2$  (Spearman) correlation of gene expression (read counts) > 0.92.

## **Functional analyses of the transcriptome data**

To determine the category to which genes belonged and discover the pathways related to the differentially expressed genes in Studies I and III, enrichment analysis was performed with the GO<sup>117</sup> and Wiki pathways databases, by the WEB-based gene set analysis toolkit v2.<sup>137</sup> In Study III, however, the Database for Annotation, Visualization and Integrated discovery (DAVID)<sup>119</sup> was used instead.

## **PROTEIN EXPRESSION ANALYSES (STUDIES I-III)**

### **Flow cytometry**

In Study I flow cytometry was applied to quantify expression of the TLR2 protein in TNF- $\alpha$ -stimulated gingival fibroblasts. The cells were seeded into Petri dishes and grown as described above, incubated with TNF- $\alpha$ , collected by trypsinization and stained with a monoclonal anti-human TLR2 antibody, followed by a secondary goat anti-mouse IgG antibody labeled with fluorescein isothiocyanate. After resuspension of the cells in phosphate-buffered saline, cell surface expression of TLR2 was assessed in a FACSCalibur™ flow cytometer.

### **Immunohistochemical analyses**

To determine whether the elevated levels of interferon regulatory factor 4 (IRF4), CCL18, mucin 4 (MUC4) and MMP7 mRNA observed in Studies II and III corresponded to changes at the protein level, we performed immunohistochemical analyses using commercially available antibodies of appropriate specificity. For additional details see Papers II and III.

## **STATISTICAL ANALYSES (STUDIES I-III)**

For statistical analyses other than those employed for the microarray and RNA-seq data (see above), we used Student's t-test (two-tailed), Fisher's exact test, and the Standard score (more commonly referred to as a Z-score). All of the experiments with cell cultures in Study I were carried out in triplicate and reproducible data from at least one of these three independent experiments are presented. The results are expressed as means  $\pm$  standard deviations and P-values less than 0.05 were considered to be statistically significant.





## RESULTS AND DISCUSSION



In the following sections a brief overview of the results from the three studies will be given and discussed in relationship to the current literature. Studies I and II have been published in peer-reviewed journals and are reproduced in their entirety in the appendix. Study III is a manuscript that has been submitted for publication.

## **STUDY I**

### **The gene expression profile of gingival fibroblasts**

Utilizing microarrays, we explored the global gene expression profile of gingival fibroblasts, the most abundant cells in gingival connective tissue, with and without stimulation by the pro-inflammatory mediator TNF- $\alpha$ . TNF- $\alpha$  induced the expression of a wide range of genes, several of which were related to signaling pathways involved in immune and inflammatory responses. The genes up-regulated significantly included those encoding TNF- $\alpha$ , TLR2, TLR3, MyD88, mitogen-activated protein kinase kinase kinase 8 (MAP3K8), inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKBKE), MAP3K, signal transducers, activators of transcription 1 (STAT1), and cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>). Enrichment analysis of the differentially expressed genes indicated significant regulation of several signaling pathways, including the TLR pathway.

### **TNF- $\alpha$ induces TLR2 expression in gingival fibroblasts**

Gingival fibroblasts, which play a pivotal role in defending against infectious diseases, express TLR2 mRNA constitutively.<sup>56</sup> This TLR2 recognizes primarily Gram-positive bacteria, peptidoglycan, and bacterial lipopeptides,<sup>138</sup> but studies have reported that TLR2 also binds LPS derived from the Gram-negative bacteria, *P.gingivalis*, commonly associated with periodontitis.<sup>139-141</sup> Binding of TLRs to their respective ligands triggers signaling events that lead to induction of various pro-inflammatory mediators, including TNF- $\alpha$ . Although TLRs normally help protect against pathogens, their aberrant expression and/or activation might destroy or compromise immune responses, a situation seen in periodontitis.

Since we had observed that TNF- $\alpha$  elevated the levels of TLR2 mRNA, which could conceivably sensitize gingival fibroblasts to pathogen-derived factors, we wished to examine the levels of the corresponding protein. After stimulating gingival fibroblasts *in vitro* with TNF- $\alpha$ , we confirmed for the first time, utilizing RT-PCR and flow cytometry, that TNF- $\alpha$  induces not only TLR2 mRNA, but also the protein.

These findings are in agreement with the reported effects of TNF- $\alpha$  on TLR2 expression by other non-immune cell types, including HUVEC cells, human airway epithelial cells, and microglia cells. Recently, Wara-aswapati and colleagues<sup>142</sup> observed that *P.gingivalis* infection induces TLR2 expression in gingival fibroblasts and that this might be a secondary effect mediated by an early autocrine action of TNF- $\alpha$ . In this same study, anti-TNF- $\alpha$  neutralizing antibodies were found to suppress the effects of *P.gingivalis* on TLR2 mRNA

expression. Accordingly, these researchers propose that the up-regulation of TLR2 by *P.gingivalis* in gingival fibroblasts is at least partially dependent on TNF- $\alpha$ .<sup>142</sup> In light of findings on the role of TLRs in the pathogenesis of various autoimmune, chronic inflammatory and infectious diseases,<sup>143,144</sup> blocking TLR2 signaling may help reduce the levels of cytokines that promote destruction of periodontal tissue.<sup>145,146</sup>

## **Signaling pathways involved in up-regulation of TLR2 by TNF- $\alpha$ in gingival fibroblasts**

Since the microarray analysis revealed that TNF- $\alpha$  up-regulates genes whose products are components of signaling pathways involved in inflammatory responses, including the JNK (MAP3K8), NF- $\kappa$ B (IKBKE), janus kinase 2/signal transducer and activator of transcription 1 (JAK2/STAT1), as well as the prostaglandin signaling pathway, the possible role of these pathways in regulation of TLR2 expression induced by TNF- $\alpha$  was explored next.

MAP3K8, a serine-threonine protein kinase member of the MAP3K family, initiates the stress-signaling JNK pathway.<sup>147</sup> We found that inhibition of this pathway attenuates the induction of both TLR2 mRNA and protein expression by TNF- $\alpha$ . The JNK pathway also appears to play a role in the chronic inflammatory disease rheumatoid arthritis. For example, in a mouse model of arthritis, pharmacological inhibition of this pathway prevented the onset of arthritis and abrogated joint swelling.<sup>148</sup> These findings together with our own indicate that inhibitors of JNK might disrupt TNF- $\alpha$ -induced signal transduction and thereby decrease expression of TLR2, providing a possible therapeutic target for controlling inflammation.

Activation of the gene encoding IKBKE leads to dissociation of NF- $\kappa$ B from its inhibitor I $\kappa$ B kinase. Inhibition of NF- $\kappa$ B decreased TLR2 expression in gingival fibroblasts. The contribution of NF- $\kappa$ B to regulation of TLR2 expression is also indicated by the finding that inhibitors of this factor diminish TNF- $\alpha$ -induced TLR2 expression in mouse microglia cells and astrocytes.<sup>149,150</sup> Thus, a potent inhibitor of this pathway might be a valuable tool for treatment of diseases involving chronic inflammation.<sup>149,150</sup>

An additional gene upregulated by TNF- $\alpha$  in our cells was the transcription factor STAT1, which is involved in the TLR signaling pathway.<sup>151</sup> Recently, one report demonstrated that STAT1 undergoes rapid phosphorylation in response to stimulation by TLR ligands, suggesting that this transcription factor plays a crucial role in TLR-induced inflammation.<sup>152</sup> We found here, however, that inhibition of the JAK2/STAT1 signaling pathway did not reduce induction of TLR2 mRNA nor protein by TNF- $\alpha$ , indicating that this pathway may not be involved.

The prostaglandin 15d-PGJ<sub>2</sub>, a known anti-inflammatory mediator,<sup>153</sup> is activated by the release of arachidonic acid via PLA<sub>2</sub>,<sup>154</sup> and in turn activates the transcription factor peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ).<sup>153,154</sup> Since the gene for cPLA<sub>2</sub> was also up-regulated by TNF- $\alpha$  in gingival fibroblasts, we further explored the role of 15d-PGJ<sub>2</sub> in TNF- $\alpha$ -induced TLR2 expression and showed that this prostaglandin inhibits this

induction. In immune cells resident in the brain, LPS-induced expression of TLR2 is down-regulated by 15d-PGJ<sub>2</sub>.<sup>155</sup> Furthermore, in a murine model of periodontitis 15d-PGJ<sub>2</sub> exerted immuno-modulatory effects, attenuating bone resorption and inflammatory responses.<sup>156</sup> These studies together with our own suggest that 15d-PGJ<sub>2</sub> may play a part in the resolution of inflammation.<sup>155,156</sup>

Even though cell cultures are commonly used as models for investigating complex diseases such as periodontitis, such systems lack important features present *in vivo*. Therefore, in the two next studies we explored the gene expression profile of gingival tissue from both patients with periodontitis and periodontally healthy subjects.

## STUDY II

In Study II, gene expression in periodontitis-affected and healthy tissues from the same individuals was examined. Both histological and immunohistochemical analysis revealed more extensive inflammation in diseased gingival tissue. Moreover, cluster analysis demonstrated that the degree of inflammation, rather than the specific individual involved appeared to influence the clustering, indicating a characteristic gene expression profile for periodontitis. Furthermore, we identified two novel genes associated with periodontitis, namely IRF4 and CCL18.

By comparing diseased and healthy gingival tissues from the same patient (n=10), we minimized the effect of individual variation. Infiltration by inflammatory cells and the numbers of CD3-positive cells, determined by histological and immunohistochemical analysis, were higher in the periodontitis-affected gingival tissue. Similarly, RNA-seq revealed higher expression of pro-inflammatory markers in most of the periodontitis-affected biopsies. These findings support the conclusion that periodontitis is characterized by an inflammatory burden.<sup>157,158</sup>

## RNA sequencing analysis

The RNA-seq data was initially analyzed using cluster analysis, revealing that the majority of the healthy gingival biopsies clustered together whereas most of the periodontitis-affected tissues grouped together. However, 3 of the 10 patients deviated from the typical clustering pattern, possibly due to the degree of inflammation in their gingival tissue or other systemic conditions.

RNA-seq comparison of genes expressed in periodontitis-affected and healthy gingival tissue revealed that those expressed solely in the periodontitis-affected tissue were related to categories/pathways indicative of inflammation, including cytokines, chemokines, and the JAK/STAT signaling pathways. In the healthy gingival tissues, on the other hand, the genes expressed were indicative of non-inflammatory pathways. These findings are consistent with the elevated increased inflammatory response observed in the periodontitis-affected gingival

tissue histologically, as well as the demonstration by our microarray analysis in Study I that TNF- $\alpha$  induces inflammatory genes in gingival fibroblasts.<sup>127,159</sup> Our results are also in agreement with earlier demonstrations that immune and inflammatory pathways dominate the patterns of gene expression connected with the pathogenesis of periodontitis and that cytokines and inflammatory mediators play critical roles in the preservation of tissue homeostasis.<sup>45,121,158</sup>

## Differential gene expression analysis

One of the essential reasons for analysing gene expression data is to identify genes that are expressed differentially in two different samples. Among the top 50 genes revealed by differential gene expression analysis to be most strongly up-regulated in periodontitis were the two novel genes IRF4 and CCL18, with overexpression of the corresponding proteins as well, confirming their possible involvement in the pathogenesis of periodontitis.

The transcription factor IRF4 has been reported to regulate expression of the pro-inflammatory cytokines IL-17 and IL-21,<sup>165,166</sup> the levels of both of which are elevated in periodontitis.<sup>45</sup> Furthermore, this transcription factor appears to be involved in other chronic inflammatory diseases as well, including T-cell-dependent chronic inflammatory diseases such as inflammatory bowel disease (IBD), with a correlation between the levels of IRF4 mRNA and production of cytokines, including IL-6 and IL-17, in inflamed colon from patients with IBD.<sup>160</sup> Thus, IRF4 may be involved in the regulation of chronic mucosal inflammation.<sup>160</sup> With respect to rheumatoid arthritis, one recent article reported that binding sites for the transcription factor IRF4 are over-represented in the promoters of genes associated with susceptibility to this disease.<sup>161</sup>

The level of chemokine CCL18, expressed by macrophages, monocytes and dendritic cells, is elevated in the synovial tissue of rheumatoid arthritis patients.<sup>162</sup> Furthermore, blockage of CCL18 expression by anti-TNF- $\alpha$ -antibodies suggests that this chemokine may represent an additional potential target for anti-TNF- $\alpha$  therapy of patients with rheumatoid arthritis.<sup>163,164</sup>

However, the potential role of the transcription factor IRF4 and chemokine CCL18 in the pathology and progression of periodontitis requires more detailed investigation.

Although our study population was small, we believe using periodontitis-affected and healthy gingival tissue from the same patient minimizes individual variation that in some cases may mask the expression of genes specific for this disease. However, the characteristics of the healthy sites in such patients may not be identical to those in periodontally healthy subjects, which could hamper the identification of susceptibility genes for periodontitis. Moreover, Study II should be considered a pilot study, in which a relatively small group was employed to evaluate the applicability of the RNA-seq technique, which was relatively new at the time, along with proper handling of the gingival tissue samples.

## STUDY III

Study III on a larger cohort of patients with periodontitis (n=62) and healthy control subjects (n=62) is, to our knowledge, the first to utilize RNA-seq for transcriptome analysis in this context. With such a large sample population it is easier to detect differentially expressed genes, since a higher number of replicates provides significantly better detection power.<sup>114</sup>

As in Study II, we initially evaluated the degree of infiltration by inflammatory cells into the gingival tissue of all subjects for subsequent comparison with the gene expression data. As expected, H&E staining demonstrated that the patients with periodontitis generally showed more severe inflammation. Macrophages, key components of the innate immune, are present in higher numbers in active periodontal lesions than inactive sites.<sup>165</sup> B cells represent approximately 18% of the cells in periodontitis lesions<sup>166</sup> and appear to contribute to the degradation of connective tissue structures in periodontitis, as well as in other chronic inflammatory diseases.<sup>167,168</sup> Utilizing immunohistochemistry, we found pronounced expression of CD20 (B cells) and CD68 (macrophages) in gingival connective tissue from patients with periodontitis, compared to healthy gingival tissues.

### Transcriptome analysis

As a first step, multivariate analysis was performed to examine the general variance of gene expression in the data set. The largest variation was associated with the degree of inflammation, and the second largest variation was associated with the origin of the samples, i.e., from patients with periodontitis or healthy control subjects. These findings indicate that even though the degree of inflammation is higher in those with periodontitis, certain patterns of gene expression distinguish periodontitis from healthy tissue independently of the degree of inflammation. Additional multivariate analysis revealed that the two genes whose expression was most distinct in the case of periodontitis and might thus serve as biomarkers for this disease were those encoding MUC4 and MMP7.

### Differentially expressed genes in periodontitis

The subsequent analysis of differentially expressed genes in Study III showed 665 genes to be up-regulated and 633 down-regulated in periodontitis in comparison to healthy control tissue. Furthermore, the results of enrichment analyses were consistent with previous studies, indicating up-regulation of immune and inflammatory responses, as well as processes related to apoptosis.<sup>121,125,169,170</sup> Up-regulation of the immune response was consistent with the histological staining indicating more pronounced inflammation in gingival tissue from patients with periodontitis. Among the down-regulated genes, the enriched biological processes included organization of collagen fibrils and the extracellular matrix as well as bone development, which could reflect the disrupted tissue homeostasis characteristic of periodontitis.

Differential expression analysis identified MUC4 and MMP7 as the two genes most pronounced in connection with periodontitis and immunohistochemical analysis showed that both the MUC4 and MMP7 proteins were also expressed at significantly higher levels in gingiva from patients with periodontitis than in healthy controls. This over-expression of MUC4 and MMP7 at both the mRNA and protein level makes them potential biomarkers for periodontitis.

MUC glycoproteins lining the apical surface of epithelial cells have been detected in saliva and are thought to contribute to the clearance of oral pathogens, by preventing them from reaching the cell surface.<sup>171,172</sup> Moreover, the level of MUC4 in human gingival tissue increases upon induction of experimental gingivitis,<sup>170</sup> and an aberrant overexpression of MUC4 has been reported in malignant epithelial cells. Thus, MUC4 has been proposed to represent an attractive target for the development of antibodies, vaccines and therapeutic inhibitors.<sup>173</sup>

The gene up-regulated to the second greatest extent in periodontitis encodes MMP7, which belongs to the MMP family of enzymes that degrade components of the extracellular matrix and basement membrane.<sup>174-176</sup> MMP7 is expressed constitutively in the epithelium, most notably in the salivary glands. Although, the overall role of MMP7 in periodontal disease remains unclear,<sup>69</sup> we show here for the first time that MMP7 is overexpressed in gingival connective tissue from patients with periodontitis. The role of other MMPs in periodontitis is indicated by the elevated levels of MMP2, MMP8, and MMP9 proteins in gingival tissues, saliva, and GCF from patients with periodontitis.<sup>77,177-181</sup>

Because of certain limitations, the gene expression data presented here must be subjected to further verification in order to understand its precise biological significance. For instance, although differential expression analysis identified genes that were up- or down-regulated in the gingival tissue, this analysis does not take into consideration possible interactions between products of the differentially expressed genes and additional investigations of such interactions should further enhance the understanding of their specific functions. Moreover, gingival biopsies are rather heterogeneous, containing a variety of cell types, including resident cells such as epithelial cells and fibroblasts, as well as infiltrating cells. This complicates interpretation of the transcriptome analysis, which originates from a mix of these cell types, the relative proportions of which may vary in different biopsies. One way to circumvent this limitation is to use techniques such as the novel RNA-seq approach, with which the gene expression in each individual cell in a tissue section can be investigated simultaneously.



## MAIN FINDINGS



## STUDY I

- In human gingival fibroblasts the cytokine TNF- $\alpha$  up-regulated the expression of a wide range of genes, as well as activating several signaling pathways involved in immune and inflammatory responses.
- Induction of TLR2 expression at both the mRNA and protein levels by TNF- $\alpha$  in gingival fibroblasts, involved the JNK and NF- $\kappa$ B signaling pathways and was attenuated by the anti-inflammatory prostaglandin 15d-PGJ<sub>2</sub>.

## STUDY II

- The degree of inflammation was more pronounced in periodontitis-affected than healthy gingival tissue from the same patient.
- The pattern of transcriptome in periodontitis-affected tissues was similar in different individuals, indicating a characteristic gene expression profile for periodontitis.
- The two novel genes, IRF4 and CCL18 were overexpressed in periodontitis-affected tissue, both at the mRNA and protein levels.

## STUDY III

- Specific B cell (CD20) and macrophage markers (CD68) were detected at higher levels in gingival connective tissue from patients with periodontitis than from healthy control subjects.
- The largest variation within the study population was associated with the degree of inflammation, and the second largest variation with the origin of the biopsies (i.e. from patients or healthy controls).
- MUC4 and MMP7 were overexpressed in periodontitis, both at the mRNA and protein levels, and accounted for the largest difference between periodontitis tissue and healthy tissues.



## **FUTURE PERSPECTIVES**



Our present characterization of the global transcriptome of periodontitis should provide valuable insights into the molecular mechanism(s) underlying the pathogenesis and progression of this disease. However, these data do not provide definitive evidence concerning whether a particular gene is involved in the process, but rather identify a broad consortium of genes and pathways that are differentially expressed in the disease and thereby likely to be involved.

In future studies several factors should be considered. First, the majority of our observations concern the transcriptome and do not necessarily reflect effects on individual functional proteins, the levels of which need to be determined. This can be done not only in the gingival tissue but also in human saliva and gingival fluid. At the same time, it must be emphasized that since periodontitis is a complex inflammatory disease involving multifactorial factors and causes it is unlikely that a single, definitive biomarker can be identified. It is more likely that a combination of biomarkers that together can differentiate between the healthy and disease state will in the future help design individual medical interventions, as well as predict treatment outcome. Another important approach involves the use of transgenic animal models, perhaps involving Knockout of genes found to be of interest here. Finally, more detailed *in vitro* studies on cultures of gingival and epithelial cells, both of which are involved in the inflammatory response against periodontal pathogens in periodontitis, could provide unique new insights.





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## Signaling pathways involved in the regulation of TNF $\alpha$ -induced toll-like receptor 2 expression in human gingival fibroblasts

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### ABSTRACT

Periodontitis is a chronic inflammatory disease characterized by a host inflammatory response against bacteria that leads to destruction of the supporting structures of the teeth. Bacterial components of pathogens in the periodontal pocket are recognized by toll-like receptors (TLRs) that trigger an inflammatory response. In this study, we investigated the effects of the pro-inflammatory cytokine tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) on TLR2 expression in human gingival fibroblasts. In addition, we examined the signaling pathways involved in the regulation of TNF $\alpha$ -induced TLR2 expression. Our results showed that TNF $\alpha$  increased TLR2 mRNA and protein expression. Microarray analysis and the inhibition of specific signaling pathways demonstrated that c-Jun N-terminal kinases (JNK) and nuclear factor kappa B (NF- $\kappa$ B) were involved in the regulation of TNF $\alpha$ -induced TLR2 expression in gingival fibroblasts. Furthermore, the prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) regulatory enzyme cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) and the anti-inflammatory prostaglandin 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), were found to regulate TLR2 mRNA expression stimulated by TNF $\alpha$ . Our findings suggest that these pathways and mediators, through the regulation of TLR2 expression in gingival fibroblasts, may be involved in the pathogenesis of periodontitis. The study provides new insights into the molecular mechanisms underlying the regulation of TLR2, implicated in the chronic inflammatory disease periodontitis.

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### 1. Introduction

Periodontitis is a chronic inflammatory disease that is initiated by the formation of a microbial biofilm in the periodontium, which subsequently triggers an inflammatory response. The host response includes the release of pro-inflammatory cytokines, chemokines, prostaglandins and proteolytic enzymes, which collectively contribute to tissue and bone destruction, characterizing periodontitis [1]. The biofilm associated with periodontitis includes pathogens of the red complex: *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* and *Treponema denticola* [1]. The pathogen-associated molecular patterns of these gram-negative bacteria are recognized by a family of pattern-recognition receptors in the innate immune

system, known as toll-like receptors (TLRs) [2,3]. To date, at least 11 members of the TLR family have been identified in humans [4]. These receptors are predominantly expressed on the surface of numerous cells of the innate immune system, including neutrophils, monocytes and dendritic cells. TLRs orchestrate a rapid innate immune response against invading pathogens [2–4].

TLR activation, with the exception of TLR3, induces the interaction between toll/interleukin-1 receptor domain-containing protein and the adaptor protein myeloid differentiation primary response gene (MyD88). MyD88 recruits protein kinase interleukin-1 receptor-associated kinase to induce the mitogen-activated protein kinase kinase kinase (MAP3K). MAP3K in turn activates the mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinases (JNK) and p38 as well as the transcription factor nuclear factor-kappa B (NF- $\kappa$ B) [5]. Induction of NF- $\kappa$ B activity contributes to the secretion of various key inflammatory mediators of chronic inflammatory conditions, including tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) [6,7]. In the context of TNF $\alpha$ , increased levels of TNF $\alpha$  in synovial samples have shown to be associated with the chronic inflammatory disease,

Abbreviations: *P. gingivalis*, *Porphyromonas gingivalis*; SP, SP 600125; RO, RO 106-9920; AG-490, Tyrophostin Ag 490; BPB, 4-bromophenacyl bromide; 15d-PGJ<sub>2</sub>, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>; IKK $\beta$ , inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>.

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rheumatoid arthritis [8]. With regard to periodontitis, enhanced level of TNF $\alpha$  has been demonstrated in serum as well as in gingival crevicular fluid of patients with chronic periodontitis [9,10]. Accordingly, anti-TNF $\alpha$  therapy has been used as a successful treatment for rheumatoid arthritis and has also been reported to inhibit inflammation and decrease bone loss in experimental periodontitis [11,12].

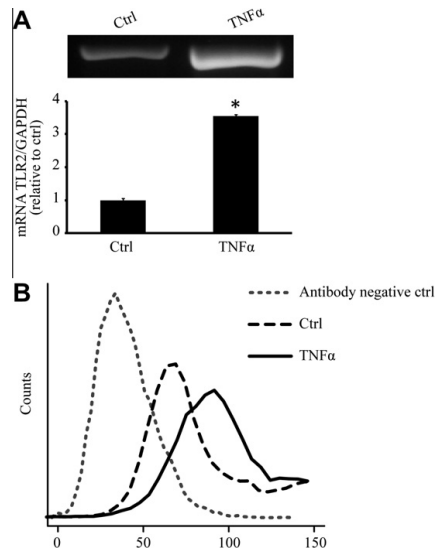
In addition to TLRs beneficial role in the host immune response, stimulation of TLR signaling, mainly via TLR2 and TLR4, may also contribute to the development of a number of diseases that are promoted by chronic inflammatory processes. For example, TLR2 has been implicated in Systemic Lupus Erythematosus (SLE), diabetes and Alzheimer's disease [13,14]. Furthermore, the administration of TLR2 and TLR4 ligands to mice leads to a significant acceleration in atherosclerosis [15,16]. In the context of periodontitis, studies have reported elevated levels of TLR2 and TLR4 in gingival tissues from patients with chronic periodontitis, compared to periodontally healthy controls [17]. Lappin et al. [18] demonstrated that the TLR2 and TLR4 agonists, Pam3-Cys-Ser-Lys4 (Pam<sub>3</sub>CSK<sub>4</sub>) and lipopolysaccharide (LPS) are elevated in saliva from patients with periodontal disease. In addition, it has been shown that gingival fibroblasts, the major cell type of the periodontal connective tissue, express TLR2 and TLR4 [19,20]. Furthermore, a study by Gutierrez-Venegas et al. [21] demonstrated that gingival fibroblasts treated with the specific TLR2 ligand; LPS from periodontitis associated bacteria *P. gingivalis*, promoted the expression of cyclooxygenase-2 (COX-2) and PGE<sub>2</sub> synthesis.

Although it is known that TLR2 is expressed in gingival tissues from patients with periodontitis, and that the expression of the receptor increases in chronic inflammation [22], the signaling pathways involved in the induction of TLR2 remains unclear. The aim of this study was to investigate the expression and regulation of TLR2 in human gingival fibroblasts stimulated with the pro-inflammatory cytokine TNF $\alpha$ . Additionally, a global gene expression profile utilizing microarray technology, was used to explore the involvement of different signal transduction pathways in the regulation of TNF $\alpha$ -induced TLR2 expression in gingival fibroblasts.

## 2. Materials and methods

### 2.1. Cell cultures of gingival fibroblasts

Human gingival fibroblast cultures were established from gingival biopsies obtained from five healthy subjects aged 3–12 years with no clinical signs of periodontal disease. The Regional Ethics Board in Stockholm approved the collection of biopsies. Minced pieces of gingival tissue were explanted to 25 cm<sup>2</sup> Falcon tissue culture flasks containing 5 ml of DMEM supplemented with penicillin (50 units/ml), streptomycin (50  $\mu$ g/ml) and 5% FCS (Invitrogen Life Technologies, Scotland, UK). Gingival fibroblasts were obtained by trypsinization of the primary cell outgrowth and, were grown at 37 °C with 5% CO<sub>2</sub> and routinely passaged using 0.025% trypsin in PBS containing 0.02% EDTA. Human gingival fibroblasts from passage level 4 to 10 were used in this study. The cells were seeded in 60-mm Petri dishes in DMEM supplemented with 5% FCS and cultured for 24 h at 37 °C. Thereafter, the cell layers were rinsed with serum-free DMEM followed by the addition of 3.0 ml DMEM containing the pro-inflammatory cytokine TNF $\alpha$  (20 ng/ml) (R&D Systems, Minneapolis, MN, USA) in the absence or presence of different test substances including SP 600125 (10  $\mu$ M) (SP; Sigma–Aldrich), RO 106-9920 (4  $\mu$ M) (RO; Tocris Bioscience, Bristol, UK), Tyrphostin AG 490 (8  $\mu$ M) (AG-490; R&D Systems), 4-bromophenacyl bromide (5  $\mu$ M) (BPB; Sigma–Aldrich), celecoxib (10  $\mu$ M) (Cayman Chemical, Ann Arbor, MI, USA) exogenous PGE<sub>2</sub> (5  $\mu$ M) (Sigma–Aldrich) or 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>



**Fig. 1.** TNF $\alpha$  increases TLR2 mRNA and protein expression in gingival fibroblasts. (A) The cells were stimulated with medium control or TNF $\alpha$  (20 ng/ml) for 6 h and the mRNA expression of TLR2 and GAPDH was analyzed by RT-PCR. The graph shows semi-quantification of TLR2 mRNA in relation to GAPDH, expressed as relative to control. Results are presented as mean  $\pm$  SD and the asterisk (\*) indicates a significant difference from control (ctrl) cells ( $p < 0.05$ ). (B) The protein expression of TLR2 in cells stimulated with TNF $\alpha$  for 24 h and analyzed by flow cytometry using TLR2 specific antibody. All analyses were performed in triplicates and the results shown are representative of three separate experiments.

(5  $\mu$ M) (15d-PGJ<sub>2</sub>; Cayman). Control cells were treated with culture medium only. After an incubation period the cell monolayer was washed twice with PBS and cells were collected for oligonucleotide microarray, reverse transcriptase-PCR (RT-PCR) or flow cytometric analysis.

### 2.2. RNA extraction

The cells were treated with TNF $\alpha$  (20 ng/ml) for 1, 3 or 6 h for microarray analysis and with TNF $\alpha$  in the presence or absence of test substances for 6 h for RT-PCR analysis. After incubation, cells were immediately frozen in liquid nitrogen and then stored at –70 °C for subsequent isolation of total RNA. RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and quantified spectrophotometrically at 260/280 nm. The RNA quality was assessed using the RNA 6000 Nano LabChip kit of the Bioanalyzer system (Agilent Technologies, Palo Alto, CA, USA).

### 2.3. Design of microarray experiments

The experimental design of the microarray study was set up as a time-course factorial design with the time points 1, 3 and 6 h to allow identification of TNF $\alpha$ -induced gene expression changes over time [24]. Each sample was measured four times in different comparisons, and the same design was repeated for fibroblasts isolated from three different subjects. For further details on the design of the microarray experiments, see Båge et al. [23].

### 2.4. Labeling and cDNA synthesis for microarray analysis

The synthesis and labeling of cDNA was performed as previously described by Lindberg et al. [25]. Briefly, Superscript III (Invitrogen)



**Table 1**List of top 10 regulated biological signaling pathways in each time comparison due to TNF $\alpha$  stimulation in gingival fibroblasts.

Time comparison	Signaling pathway	Total number of genes in the pathway	Number of regulated genes in the pathway	p Value <sup>a</sup>
1 h	TNF- $\alpha$ /NF- $\kappa$ B signaling pathway	190	21	$3.1 \times 10^{-15}$
	Apoptosis	82	12	$1.0 \times 10^{-10}$
	Toll-like receptor signaling pathway-mir	138	13	$4.4 \times 10^{-9}$
	Toll-like receptor signaling pathway	103	10	$2.0 \times 10^{-7}$
	Adipogenesis	133	11	$2.6 \times 10^{-7}$
	Id signaling pathway	51	7	$1.3 \times 10^{-6}$
	EBV LMP1 signaling	22	5	$3.4 \times 10^{-6}$
	Selenium	86	8	$4.7 \times 10^{-6}$
	Cytokines and inflammatory response (BioCarta)	23	5	$4.3 \times 10^{-6}$
	DNA damage response (only ATM dependent)	92	8	$7.7 \times 10^{-6}$
1–3 h	Apoptosis	82	22	$1.4 \times 10^{-22}$
	Toll-like receptor signaling pathway-mir	138	25	$6.5 \times 10^{-21}$
	Type II interferon signaling (IFNG)	51	15	$1.7 \times 10^{-16}$
	Toll-like receptor signaling pathway	103	18	$3.8 \times 10^{-15}$
	TNF- $\alpha$ /NF- $\kappa$ B signaling pathway	190	22	$2.5 \times 10^{-14}$
	IL-2 signaling pathway	77	15	$1.4 \times 10^{-13}$
	IL-4 signaling pathway	63	13	$2.7 \times 10^{-12}$
	Adipogenesis	133	17	$4.3 \times 10^{-12}$
	EGFR1 signaling pathway	178	19	$6.2 \times 10^{-12}$
	IL-7 signaling pathway	44	11	$1.4 \times 10^{-11}$
3–6 h	Type II interferon signaling (IFNG)	51	14	$3.6 \times 10^{-17}$
	Toll-like receptor signaling pathway	103	9	$6.5 \times 10^{-7}$
	Adipogenesis	133	10	$6.4 \times 10^{-7}$
	Toll-like receptor signaling pathway-mir	138	10	$8.9 \times 10^{-7}$
	IL-2 signaling pathway	77	7	$8.9 \times 10^{-6}$
	Androgen receptor signaling pathway	115	8	$1.5 \times 10^{-5}$
	IL-7 signaling pathway	44	5	$6.0 \times 10^{-5}$
	EGFR1 signaling pathway	178	9	$5.6 \times 10^{-5}$
	Kit receptor signaling pathway	67	6	$4.3 \times 10^{-5}$
	Apoptosis	82	6	$1.0 \times 10^{-5}$

<sup>a</sup>p Value indicates the significance of the enrichment,  $p < 0.05$ .

and random hexamer primers (Operon, Alameda CA, USA) were used for cDNA synthesizing. The reaction was terminated and RNA was hydrolyzed with EDTA and HCl. The pH was restored using NaOH and cDNA purification was performed using the MinElute Reaction Cleanup Kit (Qiagen). For washing and elution of the cDNA, 80% EtOH and NaHCO<sub>3</sub> pH 9 was used instead of supplied buffers due to the presence of Tris, which would affect the subsequent labeling reaction. Labeling was performed using the fluorescent cyanine (Cy) dyes, Cy3 and Cy5 monoreactive esters (Amersham-Biosciences, Little Chalfont, Bucks, UK). The labeling mixture was purified using the MinElute Reaction Cleanup Kit (Qiagen) according to the manufacturer's protocol. Confirmation of labeling success and measurement of the fluorophore concentrations was performed using a Nanodrop instrument (Nanodrop Technologies, Wilmington, DE, USA). The fluorophore concentrations of the labeling reactions were used to balance the sample volumes.

## 2.5. Oligonucleotide microarray

Oligonucleotide microarrays used in this study were printed at the KTH microarray core facility [26]. The 70-mer oligos originated from version 3.03 of Operons Human Genome Oligo Set, and the microarray contained 35344 features representing 28948 Entrez Gene ID:s [27] of which 17972 were unique. For additional information regarding the oligonucleotide microarray, such as the oligo set and the array manufacturing, see Båge et al. [23].

## 2.6. Hybridization

The microarrays were prehybridized with a bovine serum albumin-based buffer for 30 min, in a 42 °C water bath as previously

described [25]. After washing, the corresponding Cy3 and Cy5-labeled samples were pooled and hybridization buffer was added to the mixture. Lifter slips (Erie Scientific Company, Shelton, CT, USA) were used to contain the hybridization mixture on the array during hybridization. The arrays were hybridized overnight at 42 °C in a water bath, and after subsequent washing the arrays were immediately scanned.

## 2.7. Scanning and image processing

Scanning was performed on an Agilent G2565BA scanner (Agilent Technologies, Palo Alto, CA, USA) using a scanner resolution of 10  $\mu$ m, as previously described [25]. The software GenePix 5.1.0.0 (Axon Instruments, Foster City, CA, USA) was used to extract the raw signals from the TIFF images and to assign each spot an ID.

## 2.8. Statistical methods and low-level analysis of microarray data

The data was analyzed as previously described [23], using different packages in the software R [28]. All packages, except the KTH package [27] were available in the Bioconductor open source software project for analysis of genomic data [29]. For low-level analysis, the raw data from GenePix was read into the software R, spots were filtered for abnormal physical properties and slides were normalized using a print tip Lowess normalization [25,30]. For estimation of  $M$ -value and variance for each gene, a linear model in the Bioconductor package LIMMA (Linear models for microarray data) was set up. The  $M$ -value was the log<sub>2</sub> of the fold change, for example gene expression of TNF $\alpha$  treated cells/gene expression of control cells. The microarray data set has been deposited at the Gene Expression Omnibus (National Center for

**Table 2**

Differentially expressed genes within the TLR signaling pathway.

Time comparison	Symbol	Gene name	M-value <sup>a</sup>	Fold change	p Value <sup>b</sup>
1 h	TNF	Tumor necrosis factor (TNF superfamily, member 2)	0.79	1.7	$1.4 \times 10^{-9}$
	IFNAR2	Interferon (alpha, beta and omega) receptor 2	0.97	2.0	$3.8 \times 10^{-10}$
	IL6	Interleukin 6 (interferon, beta 2)	2.86	7.3	$4.1 \times 10^{-19}$
	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	0.28	1.2	$4.6 \times 10^{-3}$
	MAP3K8	Mitogen-activated protein kinase kinase 8	0.60	1.5	$7.4 \times 10^{-8}$
	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3.68	12.8	$2.1 \times 10^{-21}$
	IL8	Interleukin 8	4.07	16.8	$1.5 \times 10^{-10}$
	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.54	1.5	$6.2 \times 10^{-5}$
	TICAM1	Toll-like receptor adaptor molecule 1	0.29	1.2	$1.2 \times 10^{-3}$
	NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	0.51	1.4	$9.0 \times 10^{-5}$
1–3 h	TNF	Tumor necrosis factor (TNF superfamily, member 2)	−0.46	0.7	$2.1 \times 10^{-3}$
	IFNAR2	Interferon (alpha, beta and omega) receptor 2	1.25	2.4	$5.9 \times 10^{-10}$
	TICAM2	Toll-like receptor adaptor molecule 2	0.36	1.3	$1.6 \times 10^{-3}$
	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	0.39	1.3	$4.4 \times 10^{-3}$
	MAP2K1	Mitogen-activated protein kinase kinase 1	0.31	1.2	0.05
	IFNAR1	Interferon (alpha, beta and omega) receptor 1	0.46	1.4	$3.0 \times 10^{-4}$
	CD40	CD40 molecule, TNF receptor superfamily member 5	0.40	1.3	$3.6 \times 10^{-3}$
	CXCL10	Chemokine (C–X–C motif) ligand 10	2.35	5.1	$3.0 \times 10^{-4}$
	TLR3	Toll-like receptor 3	0.40	1.3	0.01
	IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	0.63	1.6	$5.1 \times 10^{-4}$
	IL6	Interleukin 6 (interferon, beta 2)	−0.79	1.0	$3.0 \times 10^{-3}$
	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (p105)	1.09	2.1	$2.2 \times 10^{-8}$
	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	−0.69	0.6	$1.3 \times 10^{-3}$
	STAT1	Signal transducer and activator of transcription 1, 91 kDa	0.66	1.6	$4.0 \times 10^{-4}$
	TRAF3	TNF receptor-associated factor 3	0.48	1.4	$3.2 \times 10^{-6}$
	PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	0.39	1.3	$5.3 \times 10^{-3}$
	NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	0.78	1.7	$1.0 \times 10^{-5}$
	RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	0.47	1.4	$1.6 \times 10^{-4}$
3–6 h	TLR2	Toll-like receptor 2	0.35	1.3	$6.4 \times 10^{-3}$
	CXCL10	Chemokine (C–X–C motif) ligand 10	2.46	5.5	$6.0 \times 10^{-5}$
	TLR3	Toll-like receptor 3	0.60	1.5	$2.6 \times 10^{-6}$
	IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	0.49	1.4	0.01
	IL6	Interleukin 6 (interferon, beta 2)	0.70	1.6	$2.0 \times 10^{-3}$
	STAT1	Signal transducer and activator of transcription 1, 91 kDa	1.38	2.6	$1.2 \times 10^{-10}$
	IRF7	Interferon regulatory factor 7	1.16	2.2	$5.1 \times 10^{-7}$
	MYD88	Myeloid differentiation primary response gene (88)	0.34	1.3	$1.3 \times 10^{-6}$
	RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	0.38	1.3	$3.4 \times 10^{-3}$

<sup>a</sup> Positive *M*-values indicate genes upregulated by TNF $\alpha$ , and negative *M*-values indicate genes down-regulated by TNF $\alpha$ .<sup>b</sup> Differential expression is defined as  $p < 0.05$ .

Biotechnology Information) with Accession No. GSE13903 [31]. Subsequently, differentially expressed genes were identified in the different contrasts of interest using a moderated *t*-test [32]. A false discovery rate algorithm was then applied to the calculated *p* values to correct for multiple testing [33]. Thereafter, differentially expressed genes were defined as genes with a *q* value  $< 0.05$  (the false discovery rate analog of a *p* value), meaning that the proportion of false positives among the differentially expressed genes was 0.05. Enrichment analysis of the data was then performed using the WikiPathways database through WEB-based Gene Set Analysis Toolkit V2 (WebGestalt) [34], to discover which intracellular pathways were regulated among the differentially expressed genes at the different time comparisons of TNF $\alpha$  treatment.

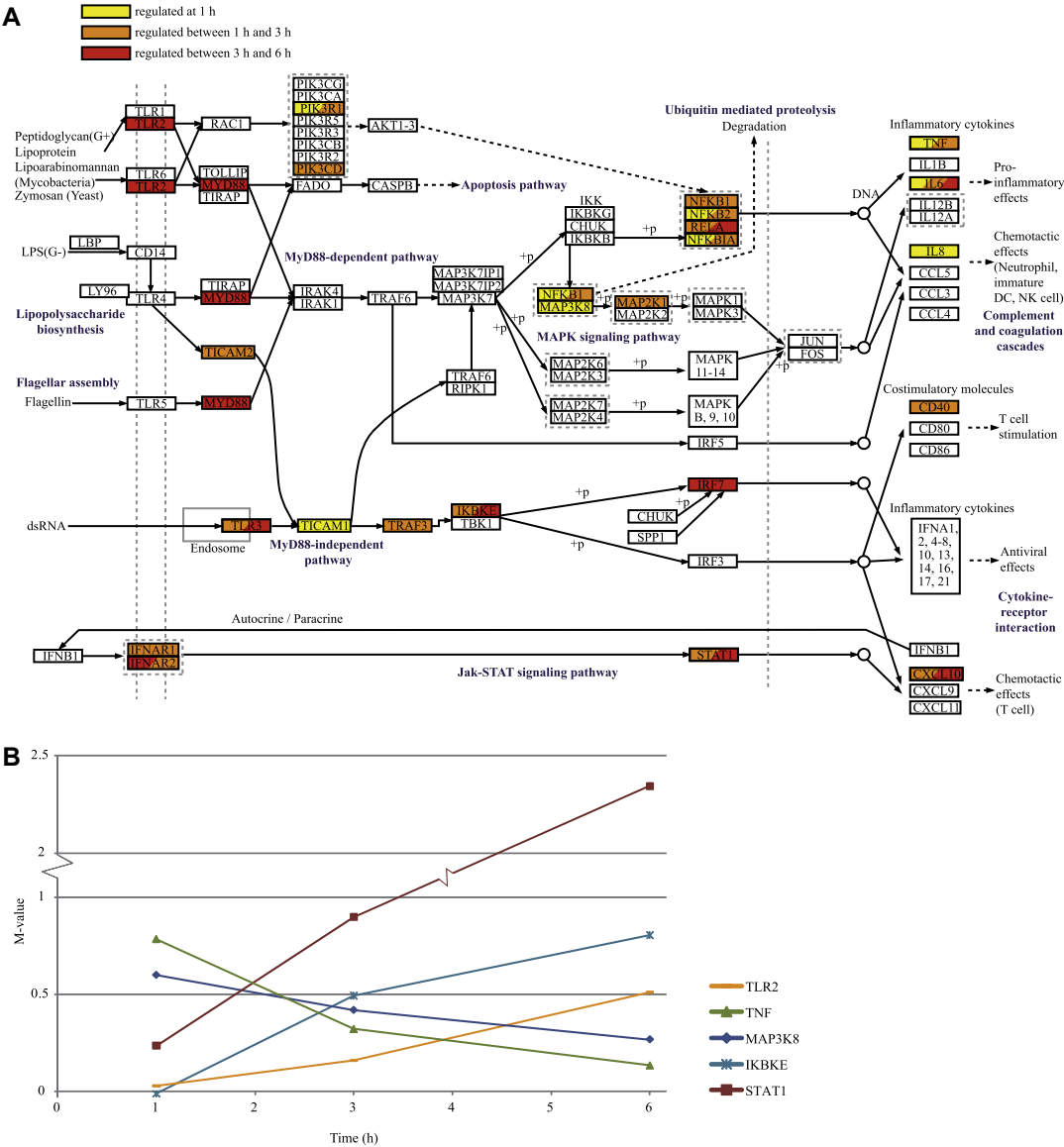
## 2.9. RT-PCR

For the reverse transcription reaction, Superscript II (Invitrogen) was used with 1.0  $\mu$ g total RNA in a total reaction volume of 20  $\mu$ l. PCR amplifications were performed using 5.0  $\mu$ l cDNA and AmpliTaq Gold DNA polymerase (Invitrogen) to a final reaction volume of 50  $\mu$ l, with specific commercial primer pairs for TLR2 (Invivogen, San Diego, CA, USA). The samples were amplified (GeneAmp PCR 9600; Applied Biosystems) for TLR2 by an initial

denaturation for 2 min at 95 °C, followed by 35 cycles of amplification (95 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min) and an additional extension step for 5 min at 72 °C. GAPDH (R&D Systems) was used as control and the reactions were performed according to the manufacturer's recommendation. For each experiment, PCR amplifications were also performed without cDNA to obtain negative controls. Ten microliters of each PCR reaction product was run on 2% agarose gels containing Gelred (Bio-nuclear Scandinavia, Stockholm, Sweden), together with 1 kb DNA-ladder standard (Invitrogen). The product sizes were 657 bp for TLR2 and 576 bp for GAPDH. The RT-PCR products were quantified using the molecular imager Chemidoc XRS system (Bio-Rad, Hercules, CA, USA) by measuring the volume intensity of the bands.

## 2.10. Flow cytometric analysis

Gingival fibroblasts were seeded into 60-mm Petri dishes and grown as described above. After treatment with TNF $\alpha$  (20 ng/ml) for 24 h, the cells were collected by trypsinization and washed three times with PBS. Thereafter, the cells were stained with anti-human monoclonal TLR2 antibody (R&D systems) followed by secondary goat anti-mouse Fluorescein Isothiocyanate-labeled antibody (DakoCytomation, Glostrup, Denmark). After washing with PBS buffer, the gingival fibroblasts were resuspended in PBS

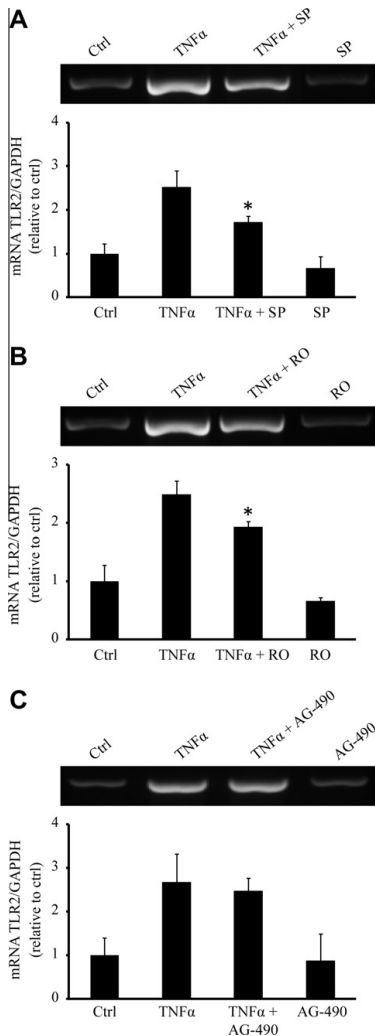


**Fig. 2.** Illustration of time-dependent cross-talk and expression of regulated genes within the TLR signaling pathway. Gingival fibroblasts were cultured with or without TNF $\alpha$  (20 ng/ $\mu$ l) for 1, 3 or 6 h and gene expression was analyzed by microarray. (A) The TLR signaling pathway according to WikiPathways [34] with the unregulated TLR7-9 removed for clarity. Genes within the TLR signaling pathway that were significantly regulated at the different time points are highlighted in yellow (1 h), orange (1–3 h) and red (3–6 h). (B) TNF $\alpha$ -stimulated time-dependent expression of the selected genes MAP3K8, IKKBE and STAT1 in comparison to TLR2 and TNF $\alpha$  from the TLR signaling pathway.

and analyzed regarding cell surface expression of TLR2 in a FACSCalibur™ flow cytometer using CellQuestPro software (Becton & Dickinson, San Jose, CA, USA). For each sample, between 10,000 and 20,000 events were acquired. The results are shown in graphs presenting the fluorescence intensity, drawn using the software R together with the package rflowcyt [28,29], and by dot-plot analyses using the CellQuestPro software (Becton & Dickinson).

2.11. Statistics for non-microarray analyses

All non-microarray experiments were performed in fibroblasts obtained from at least three subjects and reproducible data is shown as the mean  $\pm$  SD of triplicate analyses. Student's *t*-test (two-tailed) was used in the statistical analysis and *p* values less than 0.05 were considered statistically significant.



**Fig. 3.** JNK and NF- $\kappa$ B but not the JAK2/STAT1 signaling pathway regulates TNF $\alpha$ -induced TLR2 mRNA expression. Gingival fibroblasts were treated for 6 h with TNF $\alpha$  (20 ng/ml) in the presence or absence of (A) the JNK inhibitor SP (10  $\mu$ M), (B) the NF- $\kappa$ B activation inhibitor RO (4  $\mu$ M) or (C) the JAK2 inhibitor AG-490 (8  $\mu$ M). The mRNA expression of TLR2 and GAPDH was analyzed by RT-PCR. The graphs show semi-quantification of TLR2 in relation to GAPDH, expressed relative to the control. Results are presented as mean  $\pm$  SD and asterisks (\*) indicate a significant difference from cells treated with TNF $\alpha$  only ( $p < 0.05$ ). All analyses were performed in triplicates and the results shown are representative of three separate experiments.

### 3. Results

#### 3.1. TNF $\alpha$ induces TLR2 mRNA and protein expression in human gingival fibroblasts

To investigate whether the pro-inflammatory cytokine TNF $\alpha$  could induce TLR2 mRNA and protein expression in gingival fibroblasts, cells were stimulated with TNF $\alpha$  (20 ng/ml). Analysis by RT-PCR showed that TLR2 mRNA levels were elevated

( $p < 0.05$ ) by TNF $\alpha$  in 6 h cultures compared to control cells (Fig. 1A). In agreement with mRNA expression, flow cytometry showed TLR2 levels to be enhanced in response to TNF $\alpha$  (Fig. 1B).

#### 3.2. Gene expression analysis in TNF $\alpha$ -stimulated human gingival fibroblasts

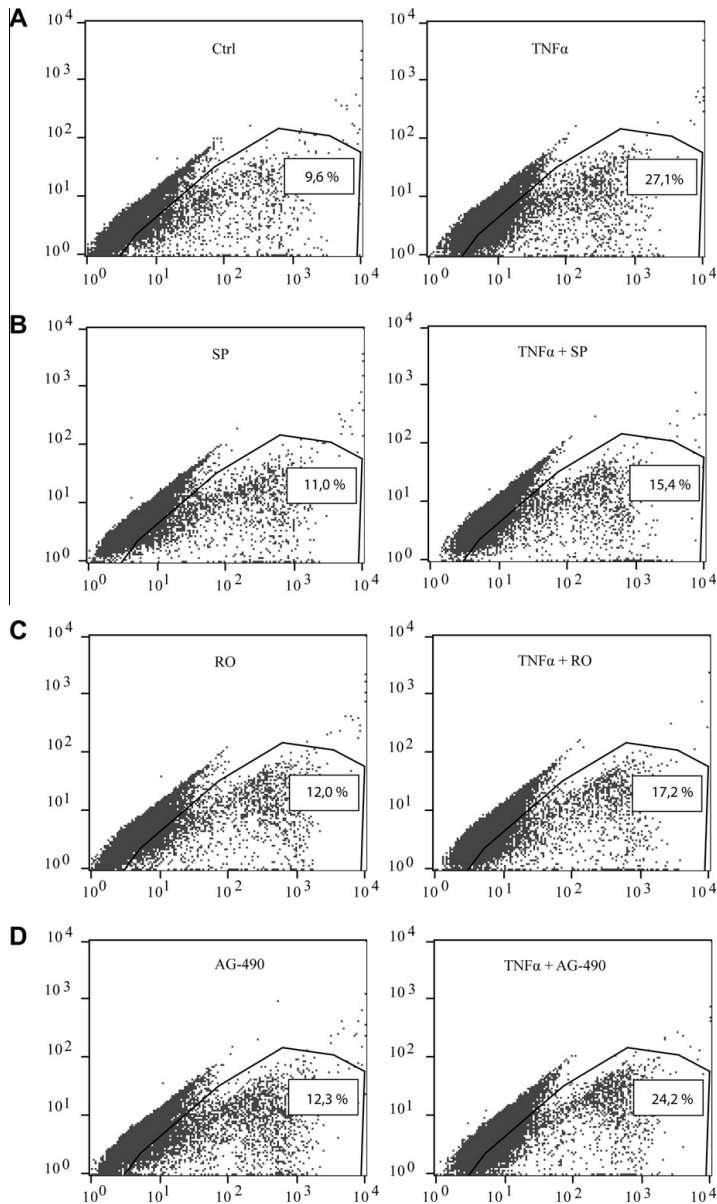
TNF $\alpha$  was used for further gene expression analysis through microarray analysis. We identified differentially expressed genes, which were defined as a false discovery rate  $< 0.05$ , in response to 1, 3 and 6 h of TNF $\alpha$  stimulation. Using a time-course factorial design, we identified genes that were differentially regulated between the selected time points with optimal statistical efficiency [23,24]. Complete lists of the differentially expressed genes within this data set, at all time comparisons of TNF $\alpha$  treatment, can be found in Supplementary File 1. Gene enrichment analysis was performed among the differentially expressed genes, across the different time comparisons, to identify TLR2 and TLR-related signaling pathways in TNF $\alpha$ -stimulated cells. The top 10 significantly regulated signaling pathways at each time comparison of TNF $\alpha$ -treatment are presented in Table 1. Among the regulated signaling pathways, the TLR signaling pathway was identified at 1 h, at the time comparisons between 1 and 3 h, as well as between 3 and 6 h of TNF $\alpha$ -stimulation (Table 1). For a complete list of all regulated pathways at each time comparison see Supplementary File 2.

Given that the TLR signaling pathway was regulated at all time comparisons of TNF $\alpha$  treatment we further investigated the differentially expressed genes within this pathway. A complete list of all differentially expressed genes within the TLR signaling pathway, including gene symbols, gene names,  $M$ -values, fold changes and  $p$  values are presented in Table 2. Among these differentially expressed genes, TLR2 was upregulated at the time comparisons between 3 and 6 h of TNF $\alpha$ -stimulation. Additional upregulated genes at this time comparison were C-X-C motif chemokine 10 (CXCL10), TLR3, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon (IKBKE), IL-6, signal transducer and activator of transcription (STAT1), interferon regulatory factor 7 (IRF7), MyD88 and v-rel reticuloendotheliosis viral oncogene homolog A (RELA). In total, the cross-talk within the TLR signaling pathway included 25 genes that were significantly regulated by TNF $\alpha$  in gingival fibroblasts at the different time comparisons (Fig. 2A). The time-dependent regulation of the genes for IKBKE, STAT1 as well as MAP3K8 in relation to TLR2 and TNF $\alpha$  (Fig. 2B), instigated further studies of the involvement of the JNK, NF- $\kappa$ B and JAK2/STAT1 signaling pathways in the regulation of TLR2 in response to TNF $\alpha$ .

#### 3.3. TNF $\alpha$ -induced TLR2 mRNA and protein expression is regulated by JNK and NF- $\kappa$ B but not by JAK2/STAT1 signaling pathway

MAP3K8 was upregulated within the TLR signaling pathway following 1 h of TNF $\alpha$  treatment, (Table 2 and Fig. 2B) and since MAP3Ks are responsible for activation of the MAPK JNK [5], we explored the involvement of the JNK signaling pathway in the regulation of TNF $\alpha$ -induced TLR2 expression. Treatment of the cells with the specific JNK inhibitor SP (10  $\mu$ M) in combination with TNF $\alpha$  reduced ( $p < 0.05$ ) TLR2 mRNA expression and the percentage of TLR2 positive cells compared to cells treated with TNF $\alpha$  alone (Figs. 3A and 4B).

Furthermore, at the time comparisons between 1 and 3 h as well as 3 and 6 h of TNF $\alpha$  treatment, the gene for IKBKE was upregulated (Table 2 and Fig. 2B). IKBKE is responsible for the activation of NF- $\kappa$ B, therefore we investigated whether the NF- $\kappa$ B signaling pathway was involved in the regulation of TNF $\alpha$ -induced TLR2 expression in gingival fibroblasts. Cells were treated with TNF $\alpha$  (20 ng/ml) in the presence or absence of the NF- $\kappa$ B activation inhibitor, RO (4  $\mu$ M). In the presence of the RO inhibitor, TLR2

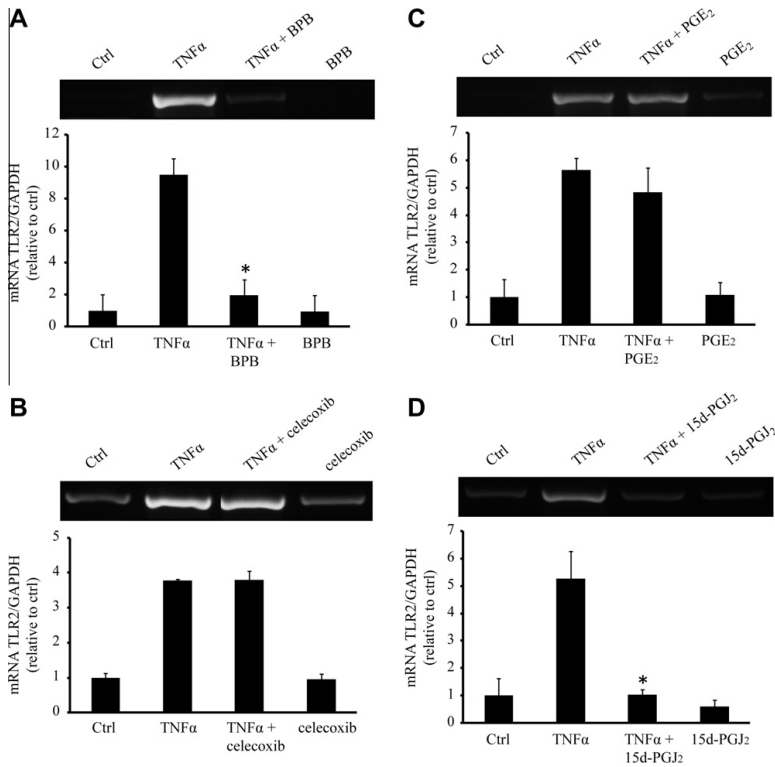


**Fig. 4.** JNK and NF- $\kappa$ B but not JAK2/STAT1 signaling pathway decrease TNF $\alpha$ -induced TLR2 positive cells. Gingival fibroblasts were treated for 24 h with (A) medium only or TNF $\alpha$  (20 ng/ml) in the presence or absence of (B) the JNK inhibitor SP (10  $\mu$ M), (C) the NF- $\kappa$ B activation inhibitor RO (4  $\mu$ M) or (D) the JAK2 inhibitor AG-490 (8  $\mu$ M). The expression of TLR2 in fibroblasts was determined by flow cytometry. The quantified data is the percentage of TLR2 positive cells obtained from the dot plots. All results are representative of three separate experiments.

mRNA and the percentage of TLR2 positive cells were diminished ( $p < 0.05$ ) compared to TNF $\alpha$  alone (Figs. 3B and 4C).

The transcription factor STAT1, also reported to be involved in the TLR signaling pathway [35], increased in response to TNF $\alpha$  at the time comparison between 3 and 6 h in similarity to TLR2 (Table 2

and Fig. 2B). We explored whether the JAK2/STAT1 pathway could be involved in modulating TNF $\alpha$ -induced TLR2 expression. Cells were treated with the specific JAK2 inhibitor AG-490 (8  $\mu$ M) in combination with TNF $\alpha$  (20 ng/ml), but the JAK2 inhibitor AG-490 did not significantly affect the stimulatory effect of TNF $\alpha$  on TLR2 mRNA



**Fig. 5.** The effect of cPLA<sub>2</sub>, 15d-PGJ<sub>2</sub>, COX-2 and exogenous PGE<sub>2</sub> on the regulation of TNFα-induced TLR2 mRNA expression. Fibroblasts were treated with TNFα (20 ng/ml), in the absence or presence of (A) the PLA<sub>2</sub> inhibitor BPB (5 μM), (B) the COX-2 inhibitor celecoxib (10 μM), (C) exogenous PGE<sub>2</sub> (5 μM) or (D) 15d-PGJ<sub>2</sub> (5 μM). After 6 h of incubation, TLR2 and GAPDH mRNA expression was analyzed by RT-PCR. The graphs show semi-quantification of TLR2 mRNA in relation to GAPDH, expressed as relative to unstimulated control (ctrl) cells. Results are presented as mean ± SD and asterisks (\*) indicate a significant difference from cells treated with TNFα alone ( $p < 0.05$ ). All analyses were performed in triplicates and the results shown are representative of three separate experiments.

expression (Fig. 3C). Likewise, AG-490 did not decrease the percentage of TLR2 positive cells increased by TNFα (Fig. 4D).

#### 3.4. Involvement of the prostaglandin pathway in the regulation of TNFα-induced TLR2 expression

Gene expression analysis revealed an upregulation of MAP3K, which is responsible for activation of the p38 signaling pathway [5]. An activation of this signaling pathway leads to phosphorylation of the enzyme cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) [36]. Our microarray analysis revealed cPLA<sub>2</sub> to be upregulated among differentially expressed genes [23]. The involvement of the enzyme cPLA<sub>2</sub> in the regulation of TLR2 expression in gingival fibroblasts was investigated using the PLA<sub>2</sub> inhibitor BPB. RT-PCR analysis revealed that treatment of the cells with BPB (5 μM) decreased ( $p < 0.05$ ) TLR2 mRNA expression induced by TNFα compared to corresponding cells treated with TNFα only (Fig. 5A).

Since PLA<sub>2</sub> is one of the key enzymes regulating the biosynthesis of prostaglandins [37], the involvement of PGE<sub>2</sub> in TLR2 expression was explored in the next series of experiments. The role of COX-2, the downstream enzyme of PLA<sub>2</sub>, which converts PLA<sub>2</sub> products to prostaglandins, was investigated using the COX-2 inhibitor celecoxib (10 μM) known to inhibit PGE<sub>2</sub> synthesis. Results showed that celecoxib did not significantly affect the mRNA expression of TLR2 induced by TNFα compared to untreated cells (Fig. 5B). Similarly,

treatment of the cells with exogenous PGE<sub>2</sub> (5 μM) did not significantly affect the expression of TLR2 induced by TNFα (Fig. 5C). In contrast, treatment of the cells with the anti-inflammatory prostaglandin, peroxisome proliferator-activated receptor γ (PPAR-γ) agonist 15d-PGJ<sub>2</sub> (5 μM) reduced ( $p < 0.05$ ) TNFα-induced TLR2 mRNA expression (Fig. 5D).

#### 4. Discussion

The present study demonstrated that TLR2 mRNA and protein expression was increased by the pro-inflammatory cytokine TNFα in human gingival fibroblasts, and that the signal transduction pathways JNK and NF-κB, and the prostaglandin mediators cPLA<sub>2</sub> and 15d-PGJ<sub>2</sub> were involved in the regulation of TNFα-induced TLR2 expression.

In the first part of the study we showed that TNFα induced TLR2 expression in gingival fibroblasts at both the mRNA and protein levels. To our knowledge, this is the first study investigating the effect of TNFα on the expression of TLR2 in gingival fibroblasts. TNFα-induced TLR2 expression has previously been reported in few other non-immune cell types, including HUVEC cells, human airway epithelial cells and microglia cells [38–40].

As a second step, we proceeded to use microarray technology to investigate the global gene expression profile over different time points of TNFα treatment in gingival fibroblasts. Here we

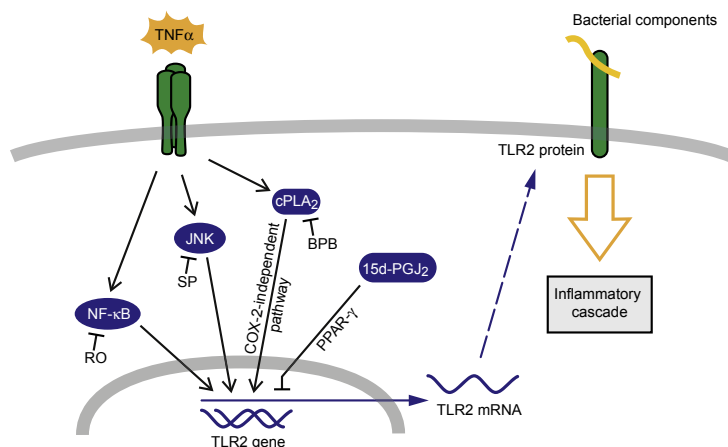


Fig. 6. Schematic illustration of signaling pathways involved in TNF $\alpha$ -induced TLR2 expression in human gingival fibroblasts.

demonstrated that TNF $\alpha$  induced a broad range of immune and inflammatory response genes in gingival fibroblasts. Enrichment analysis of the data set showed regulation of several signaling pathways involved in the immune and inflammatory response and that the TLR signaling pathway was among the top 10 regulated pathways at all time comparisons following TNF $\alpha$  treatment. To our knowledge, there are no previously published microarray studies on TNF $\alpha$ -stimulated gingival fibroblasts with special focus on TLR2 regulation. However, one microarray study has been performed on IL-1 $\beta$  stimulated gingival fibroblasts, which reports enhanced gene expression of inflammatory cytokines, transcription factors, chemokines, matrix metalloproteinases and adhesion molecules [41]. Our microarray gene expression analysis revealed several upregulated genes within the TLR signaling pathway. Among them, the gene for TLR2 was upregulated in response to TNF $\alpha$ -stimulation, which is in agreement with the RT-PCR and protein expression analyses. These findings are in concurrence with results obtained in microglia cells demonstrating that stimulation with various concentrations of TNF $\alpha$  augments the TLR2 mRNA expression in 6 h cultures [40].

A time-course factorial microarray study was set up in this study to best observe the TNF $\alpha$ -induced gene expression changes over time. Through this design we investigated the time dependent expression of the genes within the TLR signaling pathway. Our analysis showed increased expression of the gene MAP3K8 due to TNF $\alpha$  stimulation. Due to the fact that MAP3K8 is responsible for activation of the signaling pathway JNK [42], we also investigated whether JNK was involved in the regulation of TNF $\alpha$ -induced TLR2 expression in gingival fibroblasts. Our results showed that inhibition of JNK reduced TNF $\alpha$ -induced transcription of TLR2 mRNA and protein expression, suggesting the involvement of this pathway in the regulation of TNF $\alpha$ -induced TLR2 expression. The importance of the JNK pathway has also been studied in the chronic inflammatory disease rheumatoid arthritis, where pharmacologic JNK inhibition prevented the onset of arthritis and abrogated joint swelling in a mouse model of arthritis [43]. These data together with our results indicate that JNK inhibitors, by interrupting TNF $\alpha$ -induced signal transduction and by decreasing TLR2 expression, may serve as possible therapeutic targets for controlling inflammation in the tissue.

The genes for IKBKE and TLR2 were upregulated at the same time comparisons, between 3 and 6 h of TNF $\alpha$  treatment. Because

IKBKE is responsible for the dissociation of NF- $\kappa$ B and its inhibitor IKB, and in addition the TLR2 promoter contains several consensus NF- $\kappa$ B-binding motifs [44,45], the effect of NF- $\kappa$ B on the regulation of TNF $\alpha$ -induced TLR2 expression was further explored. The finding that the expression of TLR2 decreased in the presence of NF- $\kappa$ B-specific inhibitor indicates that NF- $\kappa$ B may also be involved in the regulation of TNF $\alpha$ -induced TLR2 expression in gingival fibroblasts. The contribution of NF- $\kappa$ B to the regulation of TLR2 has also been reported in mouse microglia cells and astrocytes [40,46].

STAT1 was among the upregulated genes in our microarray data set, therefore we investigated if the signaling pathway JAK2/STAT1 is involved in TLR2 expression. Our results revealed that inhibition of JAK2 did not reduce the TNF $\alpha$ -induced TLR2 mRNA or protein expression in the cells, indicating that JAK2/STAT1 may not regulate TLR2 expression in TNF $\alpha$ -stimulated gingival fibroblasts. This suggestion is in agreement with studies reporting that the JAK2/STAT1 signaling pathway is independent of the adaptor protein MyD88, which is activated by TLR2 [47,48].

Among the significantly regulated genes within the MAPK signaling pathway, the gene for cPLA<sub>2</sub> was shown to be upregulated following TNF $\alpha$  treatment. We demonstrated that inhibition of cPLA<sub>2</sub> down-regulated TNF $\alpha$ -induced TLR2 expression. Since cPLA<sub>2</sub> is one of the key enzymes that regulates the biosynthesis of PGE<sub>2</sub> from membrane phospholipids [37], we further elucidated the involvement of prostaglandins, downstream metabolites of cPLA<sub>2</sub>, in the regulation of TLR2. Therefore, experiments were performed to study the effect of the COX-2 inhibitor celecoxib and exogenous PGE<sub>2</sub>, as well as the anti-inflammatory lipid mediator 15d-PGJ<sub>2</sub> on the expression of TLR2 in gingival fibroblasts. Neither, Celecoxib, known to inhibit PGE<sub>2</sub> via the COX-2 enzyme, nor exogenous PGE<sub>2</sub> affected the expression of TLR2, suggesting a lack of a direct role for PGE<sub>2</sub> in the regulation of TLR2 expression. In contrast, the PPAR- $\gamma$  agonist, 15d-PGJ<sub>2</sub> which is activated by the release of arachidonic acid via PLA<sub>2</sub> [49] and known to mediate anti-inflammatory effects [50,51], inhibited the expression of TLR2 in gingival fibroblasts. Our results are in agreement with studies performed in brain immune resident cells, which demonstrated that the expression of LPS-induced TLR2 expression was significantly down-regulated when treated with 15d-PGJ<sub>2</sub> [52]. These findings suggest that there is a possibility that 15d-PGJ<sub>2</sub> by modulating TLR2 expression, may partly contribute to the resolution of inflammation.

In conclusion, the results showed that the inflammatory mediator TNF $\alpha$  increased TLR2 mRNA and protein expression in human gingival fibroblasts. The finding that TNF $\alpha$ , reported to be elevated in gingival fluid of patients with periodontitis [10], stimulated TLR2 expression in gingival fibroblasts suggests that TNF $\alpha$  may contribute to the upregulation of the pathogen-derived TLR2 expression in gingival tissue. Furthermore, we demonstrated that the signaling pathways JNK and NF- $\kappa$ B as well as the enzyme cPLA<sub>2</sub> and the anti-inflammatory prostaglandin 15d-PGJ<sub>2</sub> were involved in the regulation of TNF $\alpha$ -induced TLR2 expression (illustrated in Fig. 6). These results provide novel knowledge of the molecular mechanisms underlying the regulation of TLR2, which is involved in the chronic inflammatory disease periodontitis.

## Conflicts of interest

No conflicts of interest.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cyt.2011.12.008.

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# Gene Expression Profiles in Paired Gingival Biopsies from Periodontitis-Affected and Healthy Tissues Revealed by Massively Parallel Sequencing

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## Abstract

Periodontitis is a chronic inflammatory disease affecting the soft tissue and bone that surrounds the teeth. Despite extensive research, distinctive genes responsible for the disease have not been identified. The objective of this study was to elucidate transcriptome changes in periodontitis, by investigating gene expression profiles in gingival tissue obtained from periodontitis-affected and healthy gingiva from the same patient, using RNA-sequencing. Gingival biopsies were obtained from a disease-affected and a healthy site from each of 10 individuals diagnosed with periodontitis. Enrichment analysis performed among uniquely expressed genes for the periodontitis-affected and healthy tissues revealed several regulated pathways indicative of inflammation for the periodontitis-affected condition. Hierarchical clustering of the sequenced biopsies demonstrated clustering according to the degree of inflammation, as observed histologically in the biopsies, rather than clustering at the individual level. Among the top 50 upregulated genes in periodontitis-affected tissues, we investigated two genes which have not previously been demonstrated to be involved in periodontitis. These included interferon regulatory factor 4 and chemokine (C-C motif) ligand 18, which were also expressed at the protein level in gingival biopsies from patients with periodontitis. In conclusion, this study provides a first step towards a quantitative comprehensive insight into the transcriptome changes in periodontitis. We demonstrate for the first time site-specific local variation in gene expression profiles of periodontitis-affected and healthy tissues obtained from patients with periodontitis, using RNA-seq. Further, we have identified novel genes expressed in periodontitis tissues, which may constitute potential therapeutic targets for future treatment strategies of periodontitis.

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## Introduction

Periodontitis is a chronic inflammatory disease characterized by the destruction of periodontal tissue. This common disease, primarily initiated by periodontal pathogens, is an outcome of a complex interaction between periodontal microorganisms and the host inflammatory response [1]. The host response involves proinflammatory cytokines, chemokines, prostaglandins, Toll-like receptors and proteolytic enzymes, which have all been demonstrated to play an important role in the pathogenesis of periodontitis [2,3].

Studies have been performed combining *in vivo* and *in vitro* approaches to identify genes responsible for periodontitis. To date, there are a few published microarray studies investigating the gene expression profile in periodontitis. One microarray study reported no significant differences in gene expression at different pathological sites in patients with chronic and aggressive periodontitis [4], whereas Kim et al. [5] and Demmer et al. [6] showed a number of

genes that were upregulated in periodontitis compared to healthy controls. In addition, Beikler et al. [7] demonstrated that in periodontitis sites, the expression of immune and inflammatory genes was down-regulated following non-surgical therapy. With regard to *in vitro* studies, gene expression profiling has been performed on gingival fibroblasts from inflamed and healthy gingival tissues, for a limited number of inflammatory markers, such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and CD14 [8]. Furthermore, microarray analysis has also been performed on periodontal ligament cells and gingival keratinocytes [9,10]. With regard to disease susceptibility at a genomic level, one genome-wide association study (GWAS) has been conducted in patients with aggressive periodontitis showing an association between aggressive periodontitis and intronic single nucleotide polymorphism rs1537415, which is located in the glycosyltransferase gene GLT6D1 [11].

Despite research investigating periodontitis gene expression profiles through microarray analysis, specific genes responsible for the disease have not yet been found. However, the recent development of massively parallel sequencing has provided a more comprehensive and accurate tool for gene expression analysis through sequenced based assays of transcriptomes, RNA-Sequencing (RNA-Seq). This method enables analysis of the complexity of whole eukaryotic transcriptomes [12] and studies comparing RNA-Seq and microarrays have shown that RNA-Seq has less bias, a greater dynamic range, a lower frequency of false positive signals and higher reproducibility [13,14]. The aim of the present study was to investigate the general pattern of the gene expression profile in periodontitis using RNA-Seq. We also aimed to investigate the local variation in gene expression at site level, comparing periodontitis-affected and healthy gingival tissues obtained from the same patient.

Materials and Methods

Ethics Statement

The study was performed in accordance with the Declaration of Helsinki and the current legislation in Sweden and after approval from the Karolinska Institutet Ethical Research Board. The Regional Ethics Board in Stockholm approved the collection of the biopsies and informed consent was obtained from all patients.

Collection of gingival tissue samples

A total of 10 nonsmoking individuals (20 biopsies), were included in the study. Four patients in the study group had other types of diseases: patient 2 was undergoing investigations for the disease sarcoidosis, patient 3 had diabetes type-2, patient 7 had a history of osteoarthritis and patient 10 was diagnosed with asthma. All participants were examined for periodontal disease and those with a tooth site demonstrating a probing depth ≥6 mm, clinical attachment level ≥5 mm and bleeding on probing were included in the periodontitis-affected group, according to the clinical parameters previously used as indicators of periodontitis [15,16,17]. During flap surgery, two adjacent gingival biopsies with identical clinical status were harvested from a periodontal pocket affected by periodontitis. The sizes of the specimens were approximately 2×2 mm, and included the connective tissue and the epithelium. In the same subjects, two adjacent gingival biopsies with identical clinical status and of about the same size were also obtained from a clinically healthy gingival pocket. Clinically healthy pockets were defined as sites with no gingival/periodontal inflammation, no bleeding on probing, a probing depth ≤3.5 mm and a clinical attachment level ≤3.5 mm. One of the biopsies from each site was stored in RNA Later (Applied Biosystems, USA) overnight at 4°C and thereafter stored at -80°C for subsequent RNA isolation. The second biopsy from each site was used for histological and immunohistochemical analysis.

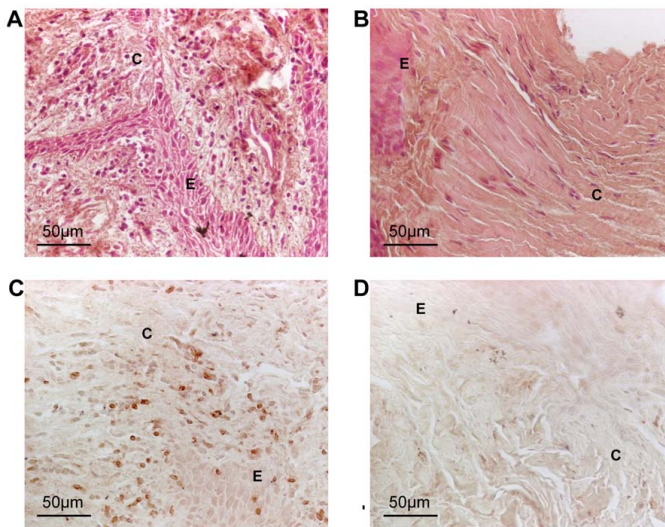
Hematoxylin-Eosin staining

Deparaffinized serial sections of gingival tissues were formalin fixed (4% neutral buffered formalin) and paraffin embedded. For assessment of orientation of the epithelium and connective tissue as well as the degree of inflammation, deparaffinized serial sections (4 μm) were prepared and sections of each biopsy were stained with Hematoxylin-Eosin (H&E). The degree of inflammatory cell infiltration was evaluated by three blinded observers, using a relative scale from 0 to 3, and statistical differences between periodontitis-affected and healthy sites were tested using the Wilcoxon signed-rank test.

Table 1. Patient characteristics and periodontal status.

Patient characteristics			Periodontitis-affected sites				Healthy sites		
Patient	Gender	Age	Probing depth (mm)	InflammationH&E (0-3) <sup>a,c</sup>	Inflammation CD3 (0-3) <sup>b,d</sup>		Probing depth (mm)	Inflammation H&E (0-3) <sup>a,c</sup>	Inflammation CD3 (0-3) <sup>b,d</sup>
1	M	52	7	3	2		3	2	0
2	M	45	8	1	1		3	1	1
3	M	52	7	3	3		3	1	1
4	F	47	7	3	3		3	1	2
5	F	37	7	2	2		3	1	1
6	M	59	7	2	3		3	2	1
7	F	66	7	2	-		3	0	-
8	M	48	6	2	2		3	0	0
9	M	42	8	3	1		2	1	1
10	F	54	6	2	1		3	1	1

<sup>a</sup>0 = no evidence of inflammatory infiltration, 1 = slight inflammatory infiltration, 2 = moderate inflammatory infiltration and 3 = severe inflammatory infiltration.  
<sup>b</sup>0 = no CD3 positive cells, 1 = low amount of CD3 positive cells, 2 = moderate amount of CD3 positive cells and 3 = high amount of CD3 positive cells and - = not enough material to perform staining.  
<sup>c</sup>Significant difference between periodontitis-affected and healthy sites (p<0.01).  
<sup>d</sup>Significant difference between periodontitis-affected and healthy sites (p<0.05).  
doi:10.1371/journal.pone.0046440.t001



**Figure 1. H&E and CD3-stained paraffin-embedded gingival biopsies obtained from one representative patient with periodontitis.** A. H&E staining of inflammatory cells in periodontitis-affected sections. B. H&E staining of inflammatory cells in healthy gingival sections. C. Staining of the T-cell marker CD3 in periodontitis-affected sections. D. Staining of the T-cell marker CD3 in healthy sections. E, epithelium, C, connective tissue. doi:10.1371/journal.pone.0046440.g001

#### Immunohistochemical stainings in gingival tissue

For staining of the T cell marker CD3, interferon regulatory factor 4 (IRF4) and chemokine (C-C motif) ligand 18 (CCL18), gingival tissues were rinsed in phosphate buffered saline (PBS) with 0.1% Saponin (PBS-Saponin buffer) for 10 min. After an antigen retrieval procedure, 10 mM Tris, 1 mM EDTA (pH 9.0) for CD3 and 0.01 M Citrate acid (pH 6.0) for IRF4 and CCL18, sections were blocked in 1%  $H_2O_2$  in PBS-Saponin for 60 min at room temperature (RT) for CD3 and for 45 min at RT for IRF4 and CCL18. Subsequently, tissues were rinsed in PBS-Saponin for 10 min and further treated with 3% bovine serum albumin (BSA) diluted in PBS-Saponin for 30 min at RT. The expression of CD3, IRF4 and CCL18 was investigated using CD3 polyclonal rabbit antibody (1 µg/ml, PBS-Saponin) from Dako Sweden AB (Stockholm, Sweden), IRF4 polyclonal rabbit antibody (0.5 µg/ml, PBS-Saponin) from Atlas antibodies (Stockholm, Sweden) and CCL18 polyclonal rabbit anti-human antibody (0.5 µg/ml, PBS-Saponin) from Sigma-Aldrich (St. Louis, MO, USA). Normal rabbit IgG from R&D systems (MN, USA) was used as negative control. After incubation with primary antibody, sections were blocked with 1% normal goat serum in PBS for 15 min. Afterwards, sections were incubated with a biotinylated secondary antibody provided in the Vectastain ABC-Elite Complex Kit (Vector labs, Burlingame, CA, USA) followed by application of the Elite ABC solution for 40 min at RT in the dark. Thereafter, sections were washed with PBS and the peroxidase activity was visualized with 0.3% (v/v) in DAB buffer containing 0.1% (v/v)  $H_2O_2$ . Finally, the slides were washed with distilled water, dehydrated through an ethanol series (70%, 95%, 99.9%) into xylene, mounted, and photographed using a light microscope. For CD3 stainings, the amount of positive cells was evaluated by three blinded observers, using a relative scale from 0 to 3, and statistical differences between periodontitis-affected and healthy biopsies were tested using the Wilcoxon signed-rank test.

#### RNA extraction

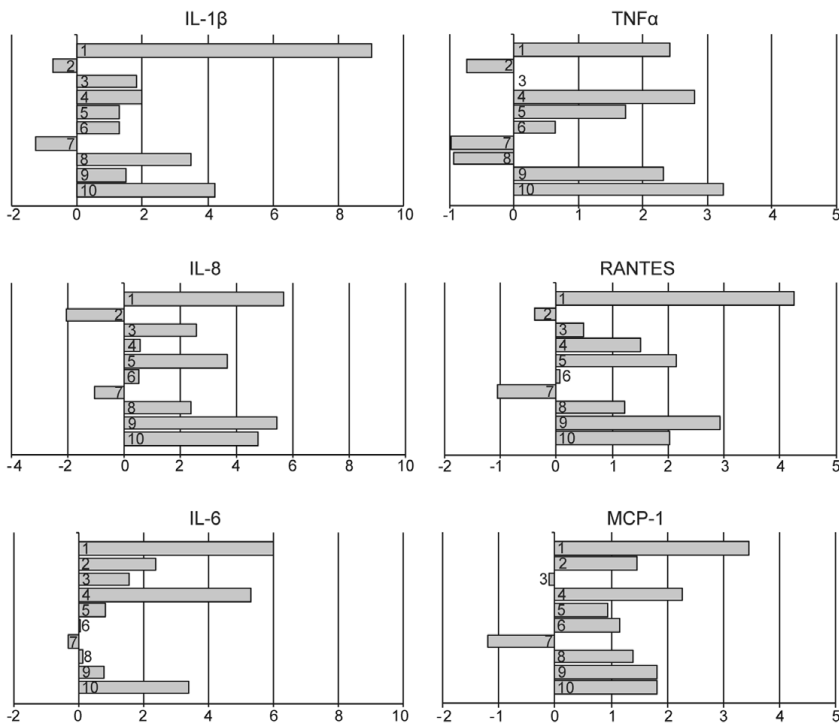
RNA was extracted from gingival biopsies using steel-bead matrix tubes and a tabletop Fast-Prep homogenizer by two sequential centrifugations for 20 s at speed 6.5 (Qiogene, Irvine, CA, USA). The RNA was purified on RNeasy Spin Columns (Qiagen, Valencia, CA, USA), treated with DNase H to ensure degradation of DNA, and thereafter eluted in RNase-free water. The average RNA yield was 15.6 µg. RNA quality was assessed using the RNA 6000 NanoLabChip Kit of the Bioanalyzer system from Agilent Technologies (Santa Clara, CA, USA).

#### Transcriptome sample preparation for sequencing

A total amount of 2–3 µg per sample was used as input material for the RNA sample preparations. All samples had RIN values above 8. The samples were bar-coded and prepared according to the protocol (Cat# RS-930-1001) from the manufacturer (Illumina, San Diego, CA, USA), as previously described by Stranneheim et al. [18]. All sample preparation reagents were taken from the Illumina mRNA Sample Preparation Kit or ordered from vendors specified in the mRNA sample preparation protocol, except for automation specific reagents: carboxylic acid beads used for precipitation; the ethanol and tetraethylene glycol (EtOH/TEG) and the Polyethylene Glycol and sodium chloride (PEG/NaCl) precipitation buffers.

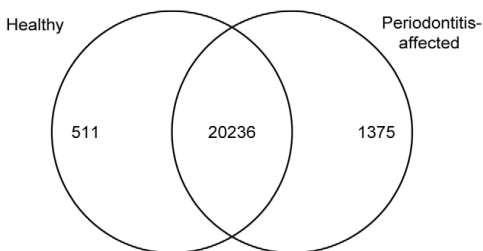
#### Clustering and sequencing

The clustering of the bar-coded samples was performed on a cBot Cluster Generation System using an Illumina HiSeq Single Read Cluster Generation Kit according to the manufacturer's instructions. The library preparations were sequenced on an Illumina HiSeq 2000 as single-reads to 100 bp. Two sequencing runs were performed according to the manufacturer's instructions where two and three lanes were used in the first sequencing and



**Figure 2. Expression of the inflammatory mediators in periodontitis-affected and healthy tissues obtained by RNA-seq.** The bars show the expression (log<sub>2</sub> fold change) pattern based on RNA-Seq reads of IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ , RANTES and MCP-1. doi:10.1371/journal.pone.0046440.g002

second sequencing run, respectively (Table S1). The runs generated a total of 402 million reads with an average of 15 million reads per sample that passed the Illumina Chastity filter; these reads were included in the study.



**Figure 3. Venn diagram of mRNA transcripts.** Venn diagram showing genes that were uniquely expressed in periodontitis-affected (1375) and healthy (511) gingival tissues. The intersection of the two circles refers to transcripts, which are expressed in both periodontitis-affected and healthy gingival tissues (20 236). doi:10.1371/journal.pone.0046440.g003

### Sequence analysis

All sequences were aligned to the human genome reference hg19 with TopHat [19,20] version 1.1.4 and Samtools [21] version 0.1.8 using TopHat standard parameters except for parameters `-solexa1.3-quals -p 8 -GTF Homo_sapiens.GRCh37.59.gtf`. Annotations from Ensembl and RefSeq, downloaded from University of California, Santa Cruz (UCSC) Genome Browser, were used to assign features to genomic positions. Sequences aligned to the human genome were assigned to features and counted using HTSeq version 0.4.6 with parameters `-m intersection-strict -s no -t exon`. The R/Bioconductor package DESeq [22] was used to call differential gene expression on read counts generated by HTSeq and to perform hierarchical clustering of samples. All biological replicates for healthy and periodontitis-affected had  $R^2$  (Spearman) correlation of gene expression (read counts) above 0.92.

### Functional analyses of gene lists using WebGestalt

Analyses of gene categories and pathways was performed using the WEB-based Gene Set Analysis Toolkit v2 (WebGestalt) [23] with parameters: Id Type: Ensembl\_gene\_stable\_id, Ref Set: Entrez Gene, Significance Level:  $p < 0.05$ , Statistics Test: Hypergeometric, MTC: BH, Minimum: 2. KEGG analysis was used for pathway enrichment analysis and the Gene ontology (GO)



**Table 2.** Enriched regulated (KEGG) biological pathways among unique genes in periodontitis-affected tissues.

Pathway	Total genes in pathway	Unique genes in pathway <sup>a</sup>	Adj <i>p</i> value <sup>b</sup>
Neuroactive ligand-receptor interaction	256	19	8.18e-10
Cytokine-cytokine receptor interaction	267	18	6.75e-09
Chemokine signaling pathway	190	10	0.0004
Intestinal immune network for IgA production	50	5	0.0014
Alanine, aspartate and glutamate metabolism	31	5	0.0022
Tyrosine metabolism	46	4	0.0103
Calcium signaling pathway	178	7	0.0160
Hedgehog signaling pathway	56	4	0.0161
Systemic lupus erythematosus	140	6	0.0168
Glycine, serine and threonine metabolism	31	3	0.0196
Jak-STAT signaling pathway	155	6	0.0229
Vascular smooth muscle contraction	115	5	0.0271
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	76	4	0.0293

<sup>a</sup>Lists of uniquely expressed genes within the enriched pathways can be found in Table S1.

<sup>b</sup>adj *p* value indicates the significance of the enrichment, (adj *p*<0.05).

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category Biological process was used for the functional annotation analysis.

## Results

### Patients and gingival tissues

A total of 10 patients, six males and four females, with a mean age of  $50 \pm 8$ , were included in the study. For each patient, a total of four gingival biopsies of about the same size were obtained from periodontitis-affected and healthy gingiva, with two biopsies from each site. Bleeding status, probing depth and degree of inflammation in the gingival tissues for each of the two gingival sites was recorded (Table 1). To assess the degree of gingival inflammation in the periodontitis-affected and healthy tissues, histological and immunohistochemistry staining was performed using H&E and anti-CD3 (Fig. 1). Scoring of the degree of inflammatory cell infiltration, assessed by H&E staining, and the amount of CD3 positive cells showed significantly higher inflammation in tissue from periodontitis-affected sites ( $p < 0.01$  for H&E and  $p < 0.05$  for CD3; Table 1).

### RNA-Sequencing

We sequenced cDNA from 10 periodontitis-affected and 10 healthy gingival tissues, with an average of 15 million reads of 100 bp in length per sample. A pairwise approach, where each periodontitis-affected biopsy had a healthy counterpart from the same individual, was used to eliminate the background noise of individual-specific gene transcription, enabling acquisition of more relevant data from the cohort. Aligning the sequence reads against the human genome yielded a median of 68% of uniquely aligned reads across all samples. The expression pattern, based on RNA-Seq reads, of well-known inflammatory mediators IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ , Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) and Monocyte Chemoattractant Protein-1 (MCP-1) were analyzed in all the tissue samples. The expression ( $\log_2$  fold change) of these mediators was shown to be higher in the majority of the periodontitis-affected gingival tissue compared to healthy gingival tissue from the same patient (Fig. 2).

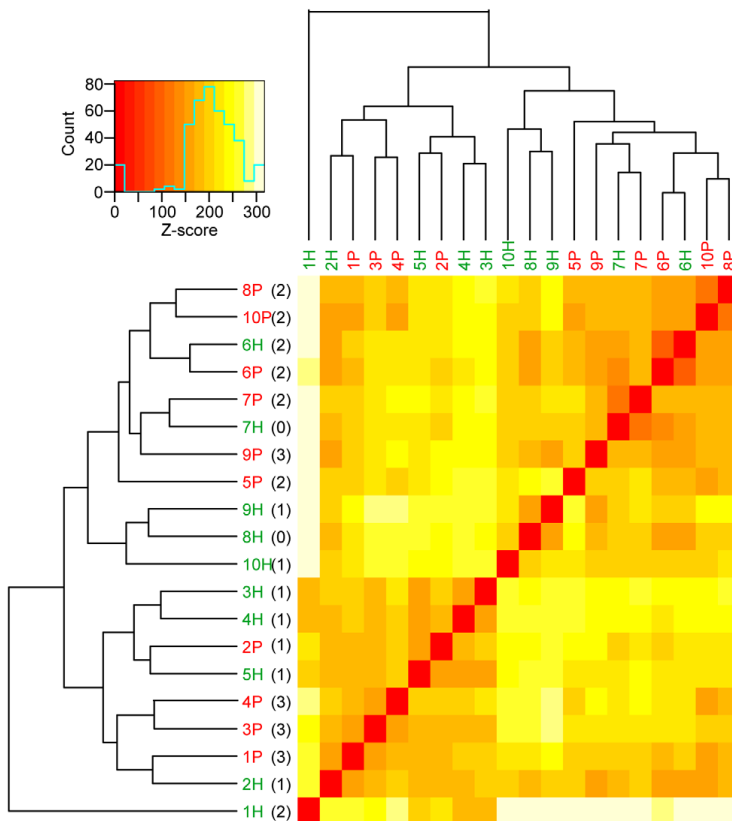
**Table 3.** Enriched regulated (KEGG) biological pathways among unique genes in healthy tissues.

Pathway	Total genes in pathway	Unique genes in pathway <sup>a</sup>	Adj <i>p</i> value <sup>b</sup>
Neuroactive ligand-receptor interaction	256	11	8.18e-10
Glycolysis/Gluconeogenesis	62	3	6.75e-09
Calcium signaling pathway	178	4	0.0004
Gap junction	90	3	0.0014
Pyruvate metabolism	40	2	0.0022
Tryptophan metabolism	40	2	0.0103

<sup>a</sup>Lists of uniquely expressed genes within the enriched pathways can be found in Table S1.

<sup>b</sup>adj *p* value indicates the significance of the enrichment, (adj *p*<0.05).

doi:10.1371/journal.pone.0046440.t003



**Figure 4. Clustering dendrogram and heatmap of periodontitis-affected and healthy biopsies.** Clustering of all samples was based on gene transcripts with a median read above three times the background noise. The length of the branch between two biopsies and the colors of the heatmap correspond to degree of similarity between the gene expression profiles. Colors can be interpreted using the scale bar. Numbers in parentheses denote the inflammation scores of the biopsies after H&E histological evaluation.  
doi:10.1371/journal.pone.0046440.g004

#### Distribution of gene transcripts between periodontitis-affected and healthy gingival tissues

A total of 22 122 different mRNA transcripts were expressed in the periodontitis-affected and healthy gingival tissue samples. Among these transcripts, 1375 were unique to the periodontitis-affected tissue samples whereas 511 genes were uniquely transcribed in healthy gingival tissues (Fig. 3). KEGG enrichment analysis using WebGestalt [24] was performed among the unique genes for the periodontitis-affected and healthy tissues which revealed several regulated pathways indicative of inflammation for the periodontitis-affected condition (Table 2 and Table S1). In contrast, in the healthy gingival tissues, regulated pathways indicated a non-inflammatory profile among the unique genes, as demonstrated in Table 3 and Table S1.

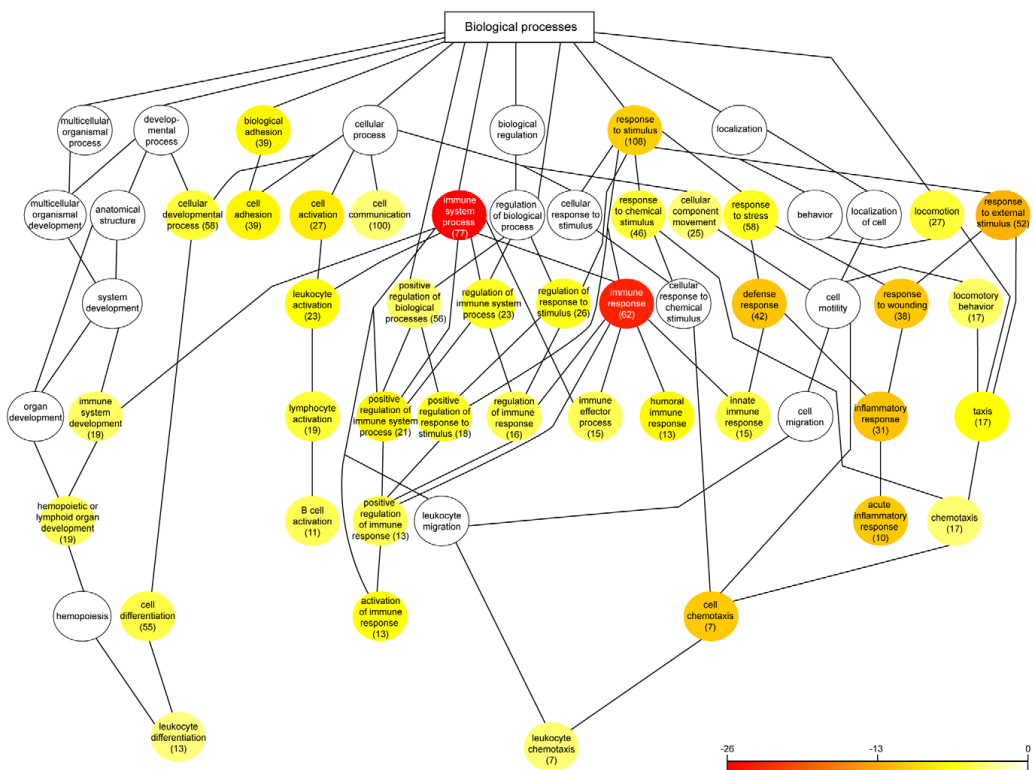
#### Clustering of biopsies

Unsupervised hierarchical clustering was performed on all gene transcripts having a median read count above a cutoff level set to 0.3 read counts per feature, to exclude expression due to spurious transcription (Fig. 4). The gingival tissues from periodontitis-

affected sites from different patients showed a more similar gene expression pattern than healthy gingival tissues from the same patient. Clustering according to individual, where the paired healthy and periodontitis-affected biopsies cluster together, was only observed for patient 6 and 7. However, the biopsies showed a general trend of clustering according to the degree of inflammation as assessed by H&E staining (Table 1), except for sample 7H, sample 2H and an outlier sample 1H, which clustered separately. There was also a trend of forming larger clusters depending on sequence run, but paired biopsies (periodontitis-affected and healthy) from each patient were always analyzed in the same sequence run.

#### Differential gene expression between periodontitis-affected and healthy gingival tissues

Differential gene expression between periodontitis-affected and healthy gingival tissues was analyzed using read counts for each gene with the DESeq package [22]. The analysis revealed a total of 453 significantly (adj  $p < 0.01$ ) differentially expressed genes. Additional analyses of genes expressed in periodontitis-affected



**Table 4.** Top 50 upregulated genes in periodontitis-affected tissue with Unigene entry.

Ensemble ID	Gene symbol	Description	Fold change	Log <sub>2</sub> fold change	p value
ENSG00000188596	C12orf63	chromosome 12 open reading frame 63	69,15	6,11	9,54e-06
ENSG00000132704	FCRL2	Fc receptor-like 2	30,36	4,92	1,39e-10
ENSG00000143297	FCRL5	Fc receptor-like 5	25,24	4,66	5,24e-30
ENSG00000116748	AMPD1	adenosine monophosphate deaminase 1 (isoform M)	24,97	4,64	5,58e-05
ENSG00000187323	DCC	deleted in colorectal carcinoma	20,69	4,37	2,37e-09
ENSG00000137265	IRF4	interferon regulatory factor 4	20,10	4,33	1,50e-32
ENSG00000167077	MEI1	meiosis inhibitor 1	16,77	4,07	3,24e-16
ENSG00000101194	SLC17A9	solute carrier family 17, member 9	14,40	3,85	2,04e-14
ENSG00000122188	LAX1	lymphocyte transmembrane adaptor 1	14,28	3,84	3,83e-20
ENSG00000110777	POU2AF1	POU class 2 associating factor 1	14,12	3,82	9,81e-26
ENSG00000124256	ZBP1	Z-DNA binding protein 1	13,76	3,78	1,60e-14
ENSG00000170476	MGC29506	hypothetical protein MGC29506	13,33	3,74	1,20e-21
ENSG00000132185	FCRLA	Fc receptor-like A	12,18	3,61	2,47e-11
ENSG0000012223	LTF	lactotransferrin	12,09	3,61	8,54e-22
ENSG00000137673	MMP7	matrix metalloproteinase 7 (matrilysin, uterine)	11,37	3,51	8,33e-18
ENSG00000163534	FCRL1	Fc receptor-like 1	11,14	3,48	1,54e-05
ENSG00000177455	CD19	CD19 molecule	11,12	3,48	2,13e-08
ENSG00000061656	SPAG4	sperm associated antigen 4	11,09	3,47	1,62e-10
ENSG00000121895	TMEM156	transmembrane protein 156	11,00	3,46	2,35e-08
ENSG0000015413	DPEP1	dipeptidase 1 (renal)	10,93	3,45	8,81e-06
ENSG00000048462	TNFRSF17	tumor necrosis factor receptor superfamily, member 17	10,43	3,38	2,71e-08
ENSG00000169962	TAS1R3	taste receptor, type 1, member 3	10,42	3,38	2,28e-06
ENSG00000102096	PIM2	pim-2 oncogene	10,09	3,34	2,43e-23
ENSG00000183508	FAM46C	family with sequence similarity 46, member C	9,94	3,31	2,19e-24
ENSG00000168081	PNOC	prepronociceptin	9,75	3,29	1,83e-07
ENSG00000099958	DERL3	Der1-like domain family, member 3	9,45	3,24	2,26e-16
ENSG00000105369	CD79A	CD79a molecule, immunoglobulin-associated alpha	9,43	3,24	1,36e-18
ENSG00000189233	C8orf80	chromosome 8 open reading frame 80	9,03	3,17	2,42e-07
ENSG00000004468	CD38	CD38 molecule	8,75	3,13	7,57e-10
ENSG00000153789	FAM92B	family with sequence similarity 92, member B	8,21	3,04	2,95e-05
ENSG00000143603	KCNN3	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	7,86	2,98	3,81e-07
ENSG00000007129	CEACAM21	carcinoembryonic antigen-related cell adhesion molecule 21	7,48	2,90	1,94e-05
ENSG00000170866	LILRA3	leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 3	7,45	2,90	0,000111577
ENSG00000129988	LBP	lipopolysaccharide binding protein	7,33	2,87	7,78e-08
ENSG00000118308	LRMP	lymphoid-restricted membrane protein	7,24	2,86	7,08e-09
ENSG00000139193	CD27	CD27 molecule	7,21	2,85	4,63e-13
ENSG00000073849	ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltransferase 1	7,11	2,83	4,37e-20
ENSG00000177272	KCNA3	potassium voltage-gated channel, shaker-related subfamily, member 3	7,07	2,82	4,64e-08
ENSG00000108405	P2RX1	purinergic receptor P2X, ligand-gated ion channel, 1	6,81	2,77	3,18e-05
ENSG00000026751	SLAMF7	SLAM family member 7	6,64	2,73	2,05e-16
ENSG00000124772	CPNE5	copine V	6,47	2,69	5,14e-10
ENSG00000132465	IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	6,41	2,68	6,97e-21
ENSG00000122224	LY9	lymphocyte antigen 9	6,39	2,68	1,71e-06
ENSG00000007312	CD79B	CD79b molecule, immunoglobulin-associated beta	6,28	2,65	1,89e-07

**Table 4.** Cont.

Ensemble ID	Gene symbol	Description	Fold change	Log <sub>2</sub> fold change	p value
ENSG00000134873	CLDN10	claudin 10	6,17	2,63	2,82e-06
ENSG00000172578	KLHL6	kelch-like 6 (Drosophila)	6,16	2,62	1,98e-11
ENSG00000196549	MME	membrane metallo-endopeptidase	6,01	2,59	2,29e-16
ENSG00000006074	CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	6,00	2,59	5,68e-10
ENSG00000173432	SAA1	serum amyloid A1	5,91	2,56	7,97e-10
ENSG00000159618	GPR114	G protein-coupled receptor 114	5,89	2,56	1,29e-05

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change and *p* value. We investigated whether there were any available reports on the involvement of these genes in periodontitis or other chronic inflammatory conditions. Among the top 50 upregulated genes, we identified a number of candidate genes, which were not previously demonstrated to be involved in periodontitis but have been shown to be associated with other chronic conditions such as rheumatoid arthritis (RA). These candidate genes included FCRL5, adenosine monophosphate deaminase 1 (AMPD1), CCL18, tumor-necrosis factor receptor superfamily 17 (TNFRSF17) and leukocyte immunoglobulin-like receptor, subfamily A (without TM domain) member 3 (LILRA3), and IRF4 which has shown to be involved in chronic inflammatory diseases such as RA and inflammatory bowel disease (IBD), (Table 5).

#### The protein expression of IRF4 and CCL18 in periodontitis-affected tissue

The expression of two of the top 50 differentially upregulated genes, IRF4 and CCL18 where further investigated at the protein level in gingival tissue samples from five additional patients with periodontitis. Immunohistochemical analysis showed that the transcription factor IRF4 and the chemokine CCL18 were expressed at the protein level in gingival tissue from patients with periodontitis (Fig. 7). IRF4 protein was expressed in cells including fibroblasts and inflammatory cells in the gingival connective tissue, as shown by morphology. For the chemokine CCL18, cellular staining of fibroblasts and inflammatory cells was observed, as well

as some diffuse extracellular staining, consistent with chemokine secretion.

#### Discussion

This study provides a novel quantitative comprehensive mapping of gene expression in gingival tissues from patients diagnosed with periodontitis, using RNA-Seq.

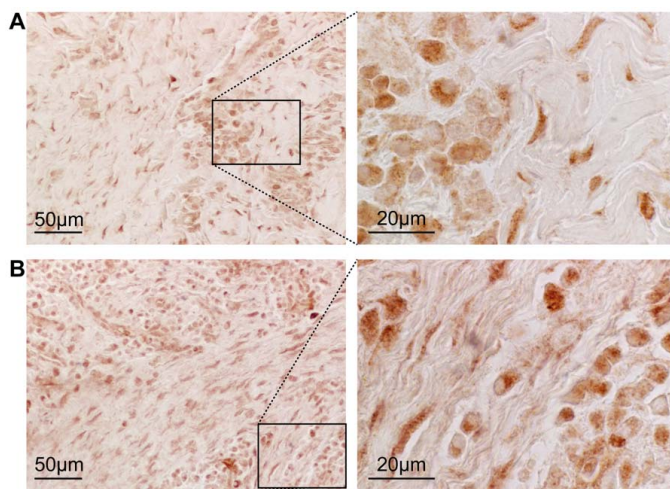
We first confirmed that the degree of inflammation was higher in periodontitis-affected gingival tissue compared to healthy tissues obtained from the same individual. Our results were based on immunohistological staining of CD3 positive cells, and further verified by RNA-Seq quantification of gene expression of the established inflammatory markers IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ , RANTES and MCP-1. These inflammatory mediators have previously been reported to be elevated in patients with periodontitis [25,26,27].

Next, we performed unsupervised clustering of the gingival tissues to get an overview of the data generated from the RNA-Seq analysis. Cluster analysis revealed that the majority of periodontitis-affected clustered together and the majority of the healthy gingival tissues also clustered together, which is in line with our results regarding inflammation in the tissues. The degree of inflammation, rather than the individual, seemed to affect the clustering, indicating a common gene expression profile for periodontitis. Our results, based on the gene expression pattern of the inflammatory markers (IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ , RANTES and MCP-1) and the immunohistochemical evaluation, confirmed

**Table 5.** Selected upregulated genes identified in periodontitis and involved in other chronic inflammatory diseases.

Ensemble ID	Gene symbol	Description	Fold change	Log <sub>2</sub> fold change	p value	Involvement in other diseases
ENSG00000143297	FCRL5	Fc receptor-like 5	25.24	4.66	5.98e-27	Rheumatoid arthritis (RA)
ENSG00000116748	AMPD1	adenosine monophosphate deaminase 1	24.97	4.64	0.0046	Rheumatoid arthritis (RA)
ENSG00000137265	IRF4	interferon regulatory factor 4	20.10	4.33	2.31e-29	Inflammatory Bowel Disease (IBD)
ENSG00000048462	TNFRSF17	tumor necrosis factor receptor superfamily, member 17	10.43	3.38	4.80e-06	Rheumatoid arthritis (RA)
ENSG00000170866	LILRA3	leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 3	7.45	2.90	0.008037	Rheumatoid arthritis (RA)
ENSG00000006074	CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	6.00	2.59	1.22e-07	Rheumatoid arthritis (RA)

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**Figure 7. Immunohistochemical stainings of IRF4 and CCL18 in the connective tissue of periodontitis-affected gingival sections. A.** Immunohistochemical staining of IRF4. **B.** Immunohistochemical staining of CCL18.  
doi:10.1371/journal.pone.0046440.g007

that the inflammation in periodontitis involves elevated levels of locally produced cytokines in the periodontium, as has been previously demonstrated [28]. However, cluster analysis revealed that three of the patients (patient no. 6, 7 and 2) deviated from the clustering pattern. For example, the healthy gingival tissue collected from patient 6 clustered with the periodontitis-affected tissue, which could be due to moderate inflammatory infiltration (H&E score 2) observed in the healthy gingival tissue. The clustering pattern in tissue from patient 7, where the healthy and diseased gingival tissue also clustered together, could be partly explained by the patient's history of osteoarthritis, which is a disease associated with elevated levels of circulating proinflammatory cytokines IL-6 and TNF $\alpha$  [29]. The cluster pattern for patient 2 differed from the rest of the patient group, which could be related to this patient undergoing investigation for the inflammatory disease sarcoidosis, and in turn might affect the systemic inflammatory response. Previous studies report that oral manifestations of sarcoidosis include aggressive destruction of the periodontium with rapid periodontal bone loss [30,31,32]. One of these studies also emphasizes the importance of patients diagnosed with sarcoidosis to be evaluated for other systemic involvements [31]. Thus, regarding our clustering pattern, it cannot be ruled out that general health differences might have some effect on the final outcome. However, the comparison of the gene expression profiles of all individuals should minimize potential interfering signals originating from single individuals affected with other diseases.

Our RNA-Seq analysis, investigating the gene expression profile in the gingival tissues showed that the genes were differentially distributed between healthy and periodontitis-affected samples. Enrichment analysis among uniquely expressed genes in the periodontitis-affected tissues showed regulated pathways indicative of inflammation, such as cytokine signaling, chemokine signaling and the JAK-STAT signaling pathway. Several cytokines such as interleukins, which are involved in periodontitis, signal through the JAK-STAT signaling pathway [33]. On the other hand, in the healthy biopsies, pathways were indicative of non-inflammatory

processes that may be involved in the maintenance of the healthy gingival tissue. Future studies should also include investigation of genes within these pathways, which may contribute to understanding, prevention and treatment of periodontitis.

Differential gene expression analyses of periodontitis-affected vs. healthy gingival tissues showed the majority of differentially expressed genes to be upregulated in the periodontitis-affected tissues. Furthermore, GO enrichment analysis among these differentially expressed genes demonstrated that most of these genes were involved in immune and inflammatory processes. This is in line with the increased inflammatory response in the tissue, and also in accordance with our previous microarray studies on inflammatory-stimulated cell cultures reporting that gene expression profiles of TNF $\alpha$ -stimulated cells show an induction of inflammatory genes [34,35].

Up to date, RNA-Seq studies aimed to identify new genes involved in the pathogenesis of periodontitis have not been reported. One *ab initio* study by Covani et al. [36] identified genes with potential roles in periodontitis, some of which have not previously been associated with the disease. However, the protein expression of these genes in periodontitis-affected tissues has not been confirmed. In our study we aimed to identify genes involved in the pathogenesis of periodontitis. Therefore, we further searched through the differentially expressed genes, focusing on the top 50 upregulated genes. Two of these 50 upregulated genes, IRF4 and CCL18, were also detected at the protein level in periodontitis affected-tissues, supporting these genes as novel finds in the pathogenesis of periodontitis. Furthermore, these two selected genes have been reported to be involved in other chronic inflammatory diseases such as RA. The transcription factor, IRF4, has been demonstrated to be involved in T-cell-dependent chronic inflammatory diseases such as IBD [37]. Mudter et al. 2011 reported a correlation between mRNA levels of IRF4 and production of cytokines such as IL-6 and IL-17 in the inflamed colon from patients with IBD, indicating that IRF4 is involved in the regulation of chronic mucosal inflammation [37]. In addition, the gene for CCL18 was upregulated in periodontitis-affected

gingival tissues. This chemokine, expressed by macrophages, monocytes and dendritic cells, has been demonstrated to be increased in synovial tissue of RA patients [38]. It has also been suggested that blockage of CCL18 expression by anti-TNF- $\alpha$  antibodies identifies CCL18 as an additional target for anti-TNF- $\alpha$  therapy in patients with RA [39,40]. Studies are currently ongoing to investigate the expression of candidate genes novel for periodontitis in a larger cohort of patients with periodontitis and healthy controls, to be able to evaluate their impact and to further explore the possible therapeutic targeting of these genes. In addition, future studies will also be performed investigating the biological significance of the down-regulated genes in periodontitis.

In conclusion, we demonstrate for the first time, using RNA-seq, profile analysis of periodontitis revealing site-specific local variation in gene expression profiles of periodontitis-affected and healthy tissues obtained from patients diagnosed with periodontitis. Furthermore, we have identified differentially expressed novel genes in gingival tissue of periodontitis. Our findings provide a first step towards a quantitative comprehensive insight into the transcriptome of gingival tissue from patients with periodontitis, to enable identification of possible diagnostic markers of periodontitis as well as potential therapeutic targets.

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## Supporting Information

**Table S1 Uniquely expressed genes within enriched pathways in periodontitis-affected and healthy gingival tissues.** (XLSX)

**Table S2 Full list of all significantly differentially expressed genes in periodontitis-affected and healthy gingival tissues.** (XLSX)

**Table S3 Gene Ontology enrichment analysis of differentially expressed genes.** (XLSX)

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## Author Contributions

Conceived and designed the experiments: HD HS TB JL TYL. Performed the experiments: HD HS ML. Analyzed the data: HD HS TB JL TYL. Contributed reagents/materials/analysis tools: ML IJ JL TYL. Wrote the paper: HD HS TB ML IJ TYL.

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III



# Transcriptome Sequencing in Periodontitis

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**Data Availability:** The authors confirm that, for approved reasons, some access restrictions apply to the data underlying the findings.

**Abbreviations:** DAVID, Database for Annotation, Visualization and Integrated Discovery; GCF, gingival crevicular fluid; H&E, Hematoxylin-Eosin; ILs, interleukins; MMPs, matrix metalloproteinases; MUC4, Mucin 4; OPLS-DA, orthogonal projections to latent structures discriminant analysis; PBS, phosphate buffered saline; PCA, principal component analysis; RNA-seq, RNA-sequencing; RA, Rheumatoid arthritis; RT, room temperature; TNF $\alpha$ , tumor necrosis factor  $\alpha$ .

## Abstract

**Background:** The chronic inflammatory disease periodontitis is characterized by destruction of periodontal tissue, and involves a complex network of cells, inflammatory mediators and signaling pathways. Despite extensive research, the complete picture of the disease is still not known. Our aim is to improve the current understanding of periodontitis, to identify candidate genes for the disease, as well as to validate their protein expression in healthy and periodontitis tissue as potential biomarkers for periodontitis.

**Results:** A total of 124 non-smoking subjects, 62 with periodontitis (mean age  $64 \pm 11$ ) and 62 healthy controls (mean age  $56 \pm 16$ ) were included in the study. RNA-sequencing (RNA-seq) analysis was performed using RNA extracted from gingival tissue biopsies. For assessment of the degree of inflammation, histological and immunohistochemical stainings were performed using H&E, anti-CD20 and anti-CD68 antibodies. The degree of inflammatory cell infiltration, evaluated in the gingival biopsies, was significantly higher in tissue obtained from patients with periodontitis compared to controls. The whole transcriptome analysis obtained by RNA-seq identified 22810 protein coding genes. Analyses of differentially expressed genes showed 665 upregulated and 633 downregulated genes in the periodontitis group. Furthermore, the categories immune- and defense response were the most significantly enriched biological processes among upregulated genes, whereas terms related to extracellular matrix/structure organization, collagen fibril organization, skeletal system development and skin/ectoderm/epidermis development were most significantly enriched among the downregulated genes. We identified two novel genes that were upregulated in periodontitis, Mucin 4 (MUC4) and matrix metalloproteinase 7 (MMP7). These genes were further confirmed at protein level in 20 gingival tissue biopsies from patients with periodontitis and 20 healthy controls.

**Conclusions:** This study reveals a comprehensive molecular profile of periodontitis, which contributes to a deeper understanding of the disease. Novel genes related to periodontitis were identified and the top two upregulated genes, MUC4 and MMP7, were also confirmed at protein level. This may lead to development of diagnostic biomarkers and therapeutic targets for periodontitis, which have the possibility to be applied in a clinical setting in the future.

## Introduction

Periodontitis is a chronic inflammatory disease characterized by tissue and bone destruction, which ultimately leads to tooth-loss. The disease is initiated when oral pathogens gain access to the gingival tissue and stimulate a host immune and inflammatory response. In susceptible individuals, inflammation fails to resolve and evolves into a chronic state, which causes destruction of the tooth-supporting structures including the gingival tissue and the underlying alveolar bone. The individual susceptibility for the disease is a result of the interactions between multiple genes and environmental factors, such as smoking or stress [1].

The hallmarks of periodontitis focus on host inflammatory events, including the release and activation of inflammatory mediators and cytokines such as interleukins (ILs) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), as well as proteolytic enzymes, such as matrix metalloproteinases (MMPs). The chronic inflammatory process in the gingival tissue is also characterized by infiltration of inflammatory cell subsets, such as cytotoxic T lymphocytes, B lymphocytes, and macrophages [2,3]. Studies have investigated the levels of IL1 $\beta$ , TNF $\alpha$  and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in gingival crevicular fluid (GCF), a serum and local-tissue exudate that provides an accurate representation of tissue and serum concentrations of inflammatory mediators [4]. These studies showed that IL1 $\beta$ , TNF $\alpha$  and PGE<sub>2</sub> levels were increased in GCF collected from periodontitis sites compared with clinically healthy sites [5,6]. In addition, the proteolytic enzymes MMPs, especially MMP2, MMP8 and MMP9 have been associated with periodontitis at protein level, in gingival tissues as well as in GCF and saliva [7-10].

Regarding the gingival tissue transcriptome in patients with periodontal disease, several studies have been performed. Demmer et al. [11] compared transcriptome from healthy and diseased papillae from patients with periodontitis. The genes identified to be upregulated in periodontitis were associated with apoptosis, antimicrobial humoral response, antigen presentation, regulation of metabolic processes, signal transduction and angiogenesis. Becker

et al. [12] showed apoptosis and proliferation processes to be most upregulated in periodontitis compared to healthy controls. Another study, focusing on inflammatory related genes, observed differences regarding gene expression involved in tissue damage and repair in periodontal tissues before and after periodontal treatment [13], while Offenbacher et al. [14] identified immune response genes as the most dominant expression pathway during induction and resolution of experimental gingivitis. The above-mentioned studies used microarray technology for the transcriptome profiling of periodontitis. Studies comparing RNA-seq versus microarray have shown RNA-seq to have higher sensitivity, wider dynamic range and lower technical variation than microarrays [15-17]. Our group has previously used RNA-seq to identify differentially expressed genes when comparing periodontitis and healthy gingival tissue obtained from the same patient. The results from the study showed that the upregulated genes were mainly indicative of immune and inflammatory responses [18].

Despite previous studies on periodontitis, the complete picture of the disease is still not understood. In this study, we therefore aim to map the gene expression pattern in periodontitis. Because of the advantages of RNA-seq compared to microarrays, we will use RNA-seq, and, include a large cohort of participants consisting of patients with periodontitis and healthy subjects to enhance the power of detection of differentially expressed genes. Additionally, we aim to validate the identified differentially expressed genes at the protein level in gingival tissue biopsies from patients with periodontitis as well as healthy tissues in order to find potential biomarkers and future therapeutic targets for periodontitis.

## **Methods**

### **Ethics Statement**

The study was performed in accordance with the Declaration of Helsinki and the current legislation in Sweden and after approval from the Karolinska Institutet Ethical Research Board. The collection of human gingival tissue samples and the experimental procedures for the study was approved by the Regional Ethics Board in Stockholm, and informed consent was obtained from all participants.

### **Subjects and collection of gingival biopsies**

A total number of 144 participants, 71 patients with periodontitis and 73 controls with no signs of periodontitis were included in the study. Patients that were included in the periodontitis group demonstrated a tooth site with a probing depth  $\geq 6$  mm, clinical attachment level  $\geq 5$  mm, as well as bleeding on probing. Subjects in the healthy control group showed no signs of periodontal disease, which was defined as sites with no gingival/periodontal inflammation, a probing depth  $\leq 3.0$  mm, a clinical attachment level  $\leq 3.5$  mm, and no bleeding on probing. The healthy controls were all undergoing implant surgery due to tooth loss for other reasons e.g. caries, accidents etc. giving us a possibility to harvest a biopsy at the same time. Two gingival biopsies of similar size were harvested from the same site from each subject. One of the biopsies was stored in RNA Later (Applied Biosystems, USA) overnight at 4°C and thereafter kept at -80°C for subsequent RNA isolation. The second biopsy was formalin fixed (4% neutral buffered formalin) and paraffin embedded for histological and immunohistochemical analysis.



### **Hematoxylin-Eosin staining**

Gingival biopsies were formalin fixed (4% neutral buffered formalin) and paraffin-embedded. Serial sections (4µm thick) were deparaffinized and stained with Hematoxylin-Eosin (H&E) for histological observations. The degree of inflammatory cell infiltration in the gingival tissues was evaluated by three blinded observers, using a relative scale from 0 to 3, where 0 = no evidence of inflammatory infiltration, 1 = slight inflammatory infiltration, 2 = moderate inflammatory infiltration and 3 = severe inflammatory infiltration. Z scores were calculated for each evaluation and the mean Z scores were reported for both groups.

### **Immunohistochemical staining for CD20, CD68, MUC4 and MMP7 in gingival tissue**

Gingival tissues were pre-heated for 1 h at 60°C followed by deparaffinization. After an antigen retrieval procedure, using Dako Target Retrieval-solution for CD20, CD68 and MMP7 (Dako Sweden AB), and 10 mM Sodium Citrate, 0.05% Tween 20 (pH 6.0) for MUC4, sections were blocked in 1% H<sub>2</sub>O<sub>2</sub> in phosphate buffer saline (PBS)-Saponin for 60 min in room temperature (RT). Subsequently, tissue sections were further incubated with 3 % bovine serum albumin for 30 min at RT. The expression of CD20 was investigated using CD20 mouse monoclonal antibody (diluted at 1:100) from Leica Biosystems, (Stockholm, Sweden), the expression of CD68 with mouse monoclonal antibody (diluted at 1:50) from Dako (Stockholm, Sweden). As for the expression of MUC4 and MMP7, MUC4 monoclonal mouse antibody and MMP7 monoclonal mouse antibody were used, both at the dilution 1:100 (Abcam, Cambridge, England). After incubation over night at 4°C with primary antibodies, sections were blocked with 1% normal goat serum for 15 min, and thereafter incubated with a horse radish peroxidase conjugated secondary polyclonal anti-mouse antibody (Dako Sweden AB, Stockholm, Sweden) for 40 min at RT in the dark. Afterwards, the Avidin-Biotin Complex solution (Vector laboratories, Burlingame, CA, US) was applied, for 40 min at RT in the dark. Further, sections were washed with PBS and the peroxidase activity was

visualized. Finally, the slides were dehydrated, mounted, and photographed using a light microscope. In parallel to each immunohistochemical staining, IgG was also used as a negative control, following the same protocol as for the antibodies used simultaneously (CD20, CD68, MUC4 or MMP7). The degree of MMP7 positive cells in epithelium and connective tissue was evaluated by three blinded observers, using a relative scale from 0 to 3. Z scores were calculated for each evaluation and the mean Z scores were reported. MUC4, CD20 and CD68 stained tissues were evaluated with whether there were positive cells or not in the tissue. The results from the periodontitis and healthy group were compared using Fisher's exact test.

### **RNA extraction**

RNA was extracted from the 144 biopsies using Qiagen RNeasy kit (VWR, Stockholm, Sweden). The quality of the extracted RNA was analyzed using the RNA 6000 NanoLabChip Kit of the Bioanalyzer system from Agilent Technologies (Santa Clara, CA, USA). Samples with RIN < 7 and a yield < 0.4 µg were excluded from further analysis, resulting in 123 remaining samples of which 65 were from patients with periodontitis and 64 were from healthy controls. The resulting mean RIN value was 8.8 for the periodontitis group and 8.9 for the healthy control group.

### **Library preparation and sequencing**

RNA libraries were prepared for sequencing using TruSeq Stranded mRNA Sample prep kit with 96 dual indexes (Illumina, CA, USA) according to the manufacturer's instructions with the following changes: The protocols were automated using an Agilent NGS workstation (Agilent, CA, USA) using purification steps as described in Lundin et al [19] and Borgström et al [20]. The samples were clustered on cBot and sequenced on HiSeq 2500 according to the manufacturer's instructions. Demultiplexing and conversion was performed using CASAVA v1.8.2. (Illumina, CA, USA) and the quality scale used was Sanger / phred33 / Illumina 1.8+.

The runs generated an average of 44.3 million paired-end reads per sample, of which on average 81% mapped to the human genome. Five samples failed sequencing quality control and were excluded from further analysis, resulting in 124 remaining samples, of which 62 were from patients with periodontitis and 62 were from healthy controls.

### **Sequence read alignment and analysis**

The paired-end sequencing reads were aligned to the human genome assembly, build GRCh37/hg19, with STAR version 2.3.1o using standard parameters [21]. The aligned reads were used to count the number of reads per gene with HTSeq version 0.6.1, with standard parameters except for `-s reverse` [22]. Mapping statistics were calculated from numbers obtained by running `bam_stat.py` included in `rseqc` version 2.3.6 on bamfiles.

### **PCA and OPLS-DA**

The read counts generated by HTSeq were used to perform principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) by means of partial least squares. PCA was performed in R version 3.1.0 using the `prcomp` function. The PCA model was based on the 500 most variable genes after adjustment of raw counts by trimmed mean of M-values (TMM) scaling implemented in the Bioconductor/edgeR package [25] followed by transformation into  $\log_2$  counts per million. For the PCA that was used to investigate the variation within the dataset with respect to the samples' degree of inflammation, one patient was excluded since the degree of inflammation was missing due to the lack of second biopsy. The software used for the OPLS-DA model and S-plot was Simca 13.0.3 (Umetrics, Umeå, Sweden). Data was log transformed and unit variance scaled before the OPLS-DA and the model complexity was estimated according to cross-validation. For the S-plot, pareto-scaling was used instead of unit variance scaling.

## **Differential expression analysis**

The R/Bioconductor package samr version 2.0 [23] was used to normalize data and call differential expression based on the read counts generated by HTSeq. Genes were considered to be significantly differentially expressed between the two conditions at a False Discovery Rate (FDR) < 0.01. The q-value, obtained when calling differential expression through SAMSeq in the samr package, describes the p-values adjusted to correct the errors arising from multiple testing, using an FDR approach as described by Storey et al. [28]. To get additional information about the identified genes, gene names and descriptions were retrieved through the R/Bioconductor package biomaRt [24] via the Ensembl ID:s outputted in the list with differentially expressed genes, obtained using samr. A few gene names and descriptions were not available through biomaRt, and those were instead manually fetched from [www.ensembl.org](http://www.ensembl.org), via their Ensembl ID:s.

## **GO category analysis**

The Database for Annotation, Visualization and Integrated Discovery (DAVID) [26] was used to perform gene functional annotation clustering of the upregulated and downregulated genes. Homo sapiens was used as background and the “GOTERM\_BP\_FAT” option selected. Default parameters were used when analyzing the functional annotation clustering.

## Results

### Subject characteristics

A total number of 124 participants, 62 patients with periodontitis and 62 healthy subjects, were included in the study. The characteristics of the study participants are demonstrated in Table 1. The gender distribution was similar in the periodontitis (33/29, females/males) and healthy control group (37/25, females/males). The mean age was  $64 \pm 11$  for the periodontitis group and  $56 \pm 16$  for the healthy controls. The clinical status obtained from all the participants showed that the probing depth, used to diagnose periodontal disease, was higher for the periodontitis group (8 mm) than the healthy control group ( $\leq 3$  mm). Bleeding on probing, a widely used criterion to diagnose gingival inflammation [27], was more frequent in the periodontitis group, 79% (49 of 62), compared to the healthy control group, 21% (13 of 62). The medical history and use of medications for each subject was also recorded and is displayed in Table 1. None of the subjects used nicotine or nicotine-replacement medications.

### Degree of inflammation and expression of CD20 and CD68 in gingival tissue biopsies

For assessment of the degree of gingival inflammation, histological stainings were performed in all biopsies from 124 participants using H&E. Figures 1A and 1B show representative histological staining with H&E of gingival tissue from patients with periodontitis and healthy controls. The analyses, based on evaluation of inflammatory cell infiltration in gingival tissues by three blinded observers, showed significantly ( $P < 0.01$ ) higher degree of inflammation in tissues from the periodontitis group (mean Z score 0.23; 95% confidence interval [CI]: 0.03 to 0.43) compared to the control group (mean Z score -0.24; 95% CI: -0.47 to -0.01) (Table 2). Immunohistochemical staining revealed a significantly ( $P < 0.01$ ) higher expression of CD20 and CD68 in gingival connective tissue from patients with periodontitis than healthy subjects. Figures 1C-F demonstrates representative sections expressing CD20 and CD68 in gingival tissue obtained from patients

with periodontitis (1C and 1E) and healthy controls (1D and 1F). CD20 was expressed in 85% (17/20) of biopsies from periodontitis patients, and 40% (8/20) of biopsies from controls (Table 3). Similarly, the CD68 staining showed a significant ( $P < 0.05$ ) difference between periodontitis and healthy controls, where 85% (17/20) of biopsies from periodontitis patients and 45% (9/20) of biopsies from controls showed expression of CD68 positive cell infiltration in the gingival connective tissue (Table 3).

### **Multivariate analysis of periodontitis and healthy controls based on gene expression data**

In order to identify causes of variation and potential outliers or biases within the dataset, an unsupervised PCA model was created based on the gene expression data. The PCA model revealed that the largest variation in the dataset, the first principal component (PC1), explained 40% of the variance within the dataset and the second principal component (PC2) explained 15%. The PCA resulted in the samples aligning along PC1 according to their degree of inflammation, which is shown in Figure 2A. PC2 showed a tendency to separate the samples into periodontitis and healthy controls, which is visualized in Figure 2B.

Furthermore, a supervised approach was applied to the dataset in order to identify which genes were responsible for the most pronounced differences between the two groups. An OPLS-DA model was therefore constructed after classification into periodontitis and healthy controls. A model with one predictive component and two orthogonal components was obtained, which successfully separated the samples into two groups along the first predictive component (Figure 3A). The degree of variation, the  $R^2$  value, was 0.629 and the model's predictive ability, the  $Q^2$  value, was 0.304 resulting in significant differences between the two groups. Additionally, an OPLS-DA model was used to create an S-plot in order to identify potential biomarkers (Figure 3B). The magnitude of the contribution of each gene to the separation in the OPLS-DA model ( $p[1]$ ) was plotted against the significance of that gene's

variation (p[corr]). The genes contributing most to the classification into periodontitis, with highest significance, were MUC4 and MMP7.

### **Differential gene expression and GO category enrichment analysis**

The RNA-seq analysis identified 22810 protein-coding genes. Differential expression analysis between the periodontitis and healthy control tissues revealed 1298 differentially expressed genes of which 665 were upregulated (supplementary data), and 633 downregulated (supplementary data) in periodontitis (with the q-value, an adjusted p-value, of 0.01). The upregulated and downregulated genes were used to identify enriched biological processes, using the functional annotation tool DAVID. The functional annotation clustering tool was used to cluster related groups as well as to order the clusters according to their significance, determined by the enrichment (EASE) scores. The three functional annotation clusters with the highest significance among upregulated genes included terms related to defense response/inflammatory response, response to wounding, apoptosis/cell death, and regulation of apoptosis/cell death (Figure 4A). The biological processes associated with downregulated genes included terms related to extracellular matrix/structure organization, collagen fibril organization, skeletal system development and skin/ectoderm/epidermis development (Figure 4B). The two most upregulated genes in periodontitis compared to healthy gingival tissue were MUC4 and MMP7.

### **Expression of MUC4 and MMP7 in gingival tissue of patients with periodontitis and healthy controls**

The normalized counts for MUC4 and MMP7 in periodontitis tissue and healthy controls are visualized in Figure 5A and D. Because these genes were the two most upregulated genes, immunohistochemical stainings were performed to confirm and verify the expression of MUC4 and MMP7 at protein level. Staining analyses were performed in 20 biopsies from the periodontitis group and 20 from healthy controls. Stainings showed positive expression of MUC4 in gingival epithelial cells in 18 patients of 20 (90 %), giving a significant difference with a p-value < 0.001 (Figure 5 and Table 3). In the gingival tissues from healthy controls, only 2 biopsies of 20 (10 %) showed expression of MUC4 in gingival epithelial cells (Table 4). Immunostaining experiments of MMP7 showed that the protein was significantly (p-value <0.01) higher expressed in connective tissue from patients with periodontitis than in healthy controls (Figure 5 and Table 4). The mean Z score of MMP7 expression was 0.40 (95% CI: 0.02; 0.78) for the periodontitis group and -0.41 (95% CI: -0.78; -0.04) for controls (Figure 5 and Table 4). However, there was no significant difference in expression of MMP7 in the epithelium of gingiva from periodontitis and healthy controls. The mean Z score in epithelial tissue was 0.16 (95% CI: -0.28; 0.6) for periodontitis and -0.05 (95% CI: -0.45; 0.35) for corresponding controls (Figure 5 and Table 4).



## Discussion

In this study, we have analyzed the whole transcriptome in periodontitis, using RNA-seq, and we propose two novel genes, MUC4 and MMP7, as potential biomarkers. We revealed these genes to be the most upregulated genes in periodontitis and they were additionally verified to be overexpressed also at protein level. Further, significant differences between periodontitis and healthy controls regarding overall gene expression patterns were identified as well as a number of differentially expressed genes between periodontitis and healthy controls. Notably, several of these genes were novel findings not earlier associated with periodontitis. Among these genes, biological processes related to inflammatory response and apoptosis were identified to be upregulated, while processes related to extracellular matrix organization and bone development were downregulated. The findings presented here provide a map over the transcriptome in periodontitis and reveal genes previously unknown to be involved in periodontitis.

RNA-seq is a powerful tool for addressing the pathophysiology of complex diseases, such as periodontitis. Previous studies investigating the transcriptome in periodontitis have mainly used microarray technology. Our group has in a previous study used RNA-seq to study the transcriptome in gingival tissue from patients with periodontitis, and in the current study we included a larger cohort of gingival tissue biopsies from 124 periodontitis and healthy controls to get a better detection power of differentially expressed genes [29]. Given that no study has had access to high-quality RNA-seq data from a cohort of this size, including both patients with periodontitis and healthy controls, the results from this study provide an important addition to the field of periodontal disease.

Inflammation is the main characteristic of periodontitis and we could confirm that the degree of inflammation was higher in the periodontitis group than in the healthy control group. In agreement with previous findings [30], we confirmed that the periodontitis group

exhibited a higher degree of inflammatory cell infiltration in gingival tissue compared to the healthy group, which is consistent with the disease characteristics. Additionally, the expression of CD20 and CD68, markers specific for B cell and macrophages, was higher in gingival connective tissue from patients with periodontitis than in healthy controls. However, we could, through the multivariate analysis, identify gene expression patterns separated from inflammation. Although the degree of inflammation was identified to be higher in the periodontitis group than in the control group, the results from the multivariate analysis indicate that there are gene expression patterns separating periodontitis from healthy tissue irrespective of the degree of inflammation. The genes contributing most to the gene expression pattern in periodontitis; MUC4 and MMP7, were reappearing in the univariate differential gene expression analysis, further supporting their significance in the pathogenesis of periodontitis.

Our GO term analysis revealed regulation of the biological processes immune response, inflammatory response, as well as processes indicative of apoptosis among the upregulated genes. These processes have also been reported by other groups [11,13,14,18]. The upregulation of the immune response processes was in agreement with results obtained from the histological stainings confirming a higher degree of inflammation in gingival tissue from patients with periodontitis. Among downregulated genes, biological processes associated with extracellular matrix organization, bone development and collagen fibril organization appeared. The downregulation of the above-mentioned processes is in line with the main characteristics of periodontitis, which is destruction of supporting connective tissue and alveolar bone.

The most upregulated genes in periodontitis were revealed to be MUC4 and MMP7. Our study is the first to demonstrate the overexpression of MUC4 and MMP7 in periodontitis. These genes might have potential as biomarkers for the early detection of periodontitis and

studies will be performed investigating the levels of MUC4 and MMP7 in saliva and GCF from patients with periodontitis and healthy controls, for a possible use in a clinical setting.

Gingival tissue consists of epithelium and connective tissue and comprises a heterogeneous mix of different cell types including epithelial cells, fibroblasts and infiltrating cells. This may have some limitations on the interpretation of the transcriptome analysis, because the observed gene expression originates from a mix of these cell types. Gingival tissue biopsies may have different number of cells, resulting in skewed gene expression patterns, a question that has been raised also by other groups [11]. Future studies should therefore focus on investigating the gene expression while also taking into account the heterogeneity in the tissues. Studies are ongoing using a novel RNA-seq method where the gene expression in each individual cell in a tissue section can be investigated simultaneously. This approach enables the association of gene expression to the cell type of interest, overcoming the limitations with RNA-seq of whole biopsies of heterogeneous tissues, such as gingival tissue.

In conclusion, this study provides better insights into the pathogenesis of periodontitis. We have revealed significant differences between the transcriptome profiles of periodontitis and healthy gingival tissues, as well as novel genes that may serve as potential biomarkers for the disease. This knowledge may lead us to new insights in the development of diagnostic biomarkers and therapeutics for periodontitis.

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## **Author contribution**

Conceived and designed the experiments: HD TB JL TYL. Performed the experiments: HD AL TB. Analyzed the data: AL HD TYL. Contributed reagents/materials/analysis tools: GJ JL TYL. Wrote the paper: AL HD TB GJ TYL.

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## Figure legends

**Figure 1. Histological and immunohistological staining of gingival biopsies obtained from patients with periodontitis and healthy controls.** Representative histological stainings with H&E in gingival tissue from a patient with periodontitis (A) and a healthy control (B). Representative immunohistological staining in gingival tissue with B cell marker CD20 and macrophage marker CD68 in gingival tissue obtained from a patient with periodontitis and (C and E, respectively) and a healthy control (D and F, respectively). Scale bars = 50 $\mu$ m.

**Figure 2. PCA score plots of RNA-seq samples show the largest and second largest variation in the dataset.** The two first principal components (PC1 and PC2) are plotted on the x- and y-axis. (A) PCA score plot of periodontitis tissues and healthy control tissues shows that the largest variation in the data set is caused by the degree of inflammation. Data points representing the samples are colored according to their degree of inflammation (green = 0, yellow = 1, orange = 2, red = 3). (B) The same PCA plot with data points color-coded red (periodontitis) and blue (healthy controls), showing a separation in principal component 2 between periodontitis tissues and healthy controls.

**Figure 3. Score scatter plot from OPLS-DA modeling of the gene expression data.** (A) The OPLS-DA score plot showing the between group variation, predictive component,  $t(1)$ , and within-group variation, orthogonal component,  $to(1)$  of periodontitis gingival tissues (red) and healthy controls (blue), with respect to gene expression. (B) S-plot from the OPLS-DA data showing the magnitude of each gene's contribution to the separation,  $p(1)$  against the reliability of each gene,  $p(\text{corr})$ . The genes contributing the most to the periodontitis group are highlighted in red and are, MMP7 and MUC4.

**Figure 4. GO categories enriched among upregulated and downregulated genes.**

Biological processes upregulated (A) and downregulated (B) in periodontitis were identified using the annotation tool DAVID. Related biological processes were clustered using the functional annotation clustering tool. The upregulated processes with the highest enrichment scores included terms related to defense response/inflammatory response, response to wounding, apoptosis/cell death, and regulation of apoptosis/cell death. The biological processes with the highest enrichment scores among downregulated genes included terms related to extracellular matrix/structure organization, collagen fibril organization, skeletal system development and skin/ectoderm/epidermis development

**Figure 5. mRNA and protein expression of MUC4 and MMP7 in gingival tissue biopsies**

**from periodontitis tissues and healthy controls.** (A) Boxplot demonstrating the normalized number of transcripts of MUC4 in periodontitis and healthy control tissues. (B) Representative staining for MUC4 in gingival epithelium from a patient with periodontitis and (C) a healthy control. (D) Normalized number of transcripts of MMP7 in periodontitis tissues and healthy tissues. (E) Staining for MMP7 in gingival epithelium from one representative patient with periodontitis and (F) a healthy control. Scale bars = 50µm.



**Table 1. The characteristics of the study participants, periodontitis and healthy controls.**

	Periodontitis	Healthy controls
<b>Patients (n)</b>	62	62
<b>Gender (f/m)</b>	33/29	37/25
<b>Mean age (min-max)</b>	64 (38-83)	56 (18-78)
<b>Mean probing depth (mm)</b>	8	3
<b>Bleeding on probing (n)</b>	49/62	13/62
<b>Other diseases</b>	16	4
Asthma	1	1
Chronic obstructive pulmonary disease	1	0
Diabetes type-2	2	0
Hypertension	12	1
Parkinson's disease	0	1
Rheumatoid arthritis	0	1
<b>Medication (n)</b>	13	2
Nonsteroidal anti-inflammatory drugs	3	1
Asthma medicine	3	0
Simvastatin	5	1
Diabetes medicine	2	0

**Table 2. The degree of inflammation by H&E in the gingival connective tissue biopsies obtained from patients (n=62) with periodontitis and healthy controls (n=61).**

<b>Degree of inflammation (0-3)<sup>a</sup></b>	<b>0</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>Mean Z score (95% CI)</b>
<b>Periodontitis (n)</b>	4	17	19	22	0.23 (0.03; 0.43)
<b>Healthy (n)</b>	11	21	17	12	-0.24 (-0.47; -0.01)

<sup>a</sup>0 = no evidence of inflammatory infiltration, 1 = slight inflammatory infiltration, 2 = moderate inflammatory infiltration and 3 = severe inflammatory infiltration.

**Table 3. Expression of CD20, CD68 and MUC4 in gingival tissue from patients with periodontitis (n=20) and healthy controls (n=20).**

Periodontitis				Healthy controls			
Subject	CD20	CD68	MUC4	Subject	CD20	CD68	MUC4
1	+	+	-	21	-	-	-
2	-	-	+	22	-	-	-
3	+	+	+	23	+	+	-
4	+	+	+	24	+	+	-
5	+	+	+	25	-	-	-
6	-	+	+	26	+	+	-
7	+	+	+	27	-	-	+
8	+	-	+	28	+	+	+
9	+	+	+	29	-	-	-
10	-	-	-	30	+	+	-
11	+	+	+	31	-	-	-
12	+	+	+	32	+	+	-
13	+	+	+	33	-	-	-
14	+	+	+	34	+	+	-
15	+	+	+	35	+	+	-
16	+	+	+	36	-	-	-
17	+	+	+	37	-	-	-
18	+	+	+	38	-	-	-
19	+	+	+	39	-	-	-
20	+	+	+	40	-	-	-

(+) evidence of CD20, CD68 or MUC4 expression, (-) no evidence of CD20, CD68 or MUC4 expression.

**Table 4. Expression of MMP7 in epithelium and connective tissue from patients with periodontitis (n=20) and healthy controls (n=20).**

Periodontitis			Healthy controls		
Subject	Epithelium (0-3) <sup>a</sup>	Connective tissue (0-3) <sup>a</sup>	Subject	Epithelium (0-3) <sup>a</sup>	Connective tissue (0-3) <sup>a</sup>
1	2	2	21	1	1
2	0	1	22	2	2
3	1	1	23	1	1
4	1	1	24	3	2
5	1	1	25	1	1
6	2	3	26	0	0
7	3	3	27	0	0
8	0	0	28	3	3
9	1	1	29	1	1
10	0	0	30	0	0
11	1	1	31	2	0
12	3	3	32	0	0
13	2	2	33	2	2
14	2	3	34	0	0
15	2	2	35	1	1
16	3	3	36	1	1
17	3	3	37	1	0
18	3	3	38	3	3
19	2	2	39	2	2
20	3	3	40	2	2
<b>Mean Z score (95% CI)</b>	0.16 (0.28; 0.6) <sup>b</sup>	0.40 (0.02; 0.78) <sup>c</sup>	<b>Mean Z score (95% CI)</b>	-0.05 (-0.45; 0.35) <sup>b</sup>	-0.41 (-0.78; -0.04) <sup>c</sup>

<sup>a</sup>0 = no evidence of inflammatory infiltration, 1 = slight inflammatory infiltration, 2 = moderate inflammatory infiltration and 3 = severe inflammatory infiltration. <sup>b</sup>Significance of difference between periodontitis and healthy controls, p = 0.49. <sup>c</sup>Significance of difference between periodontitis and healthy controls, p =  $4.8 \times 10^{-3}$ .

Figure 1.

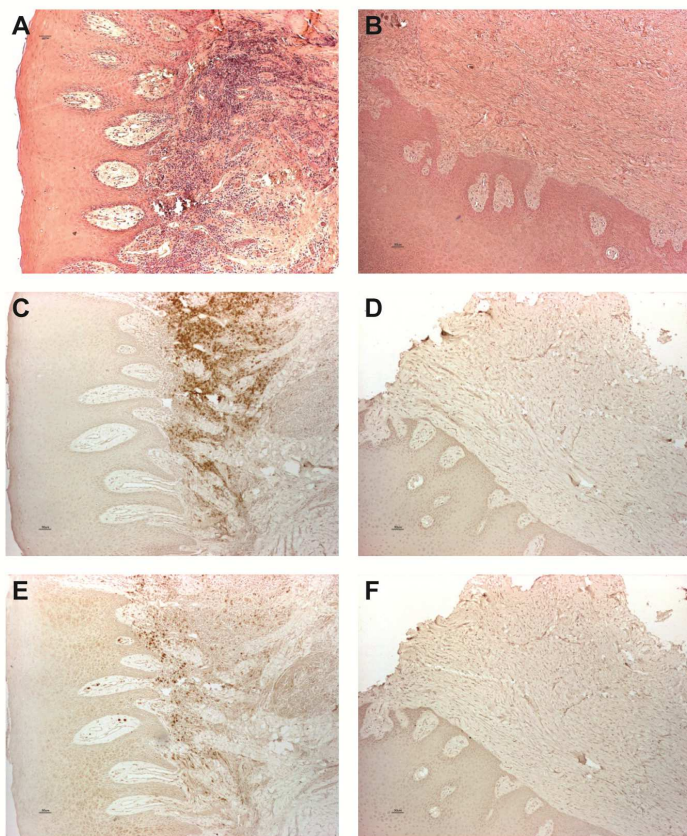


Figure 2.

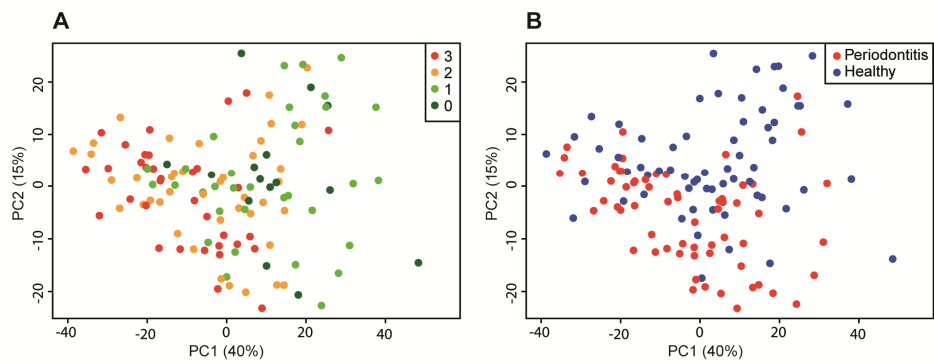


Figure 3.

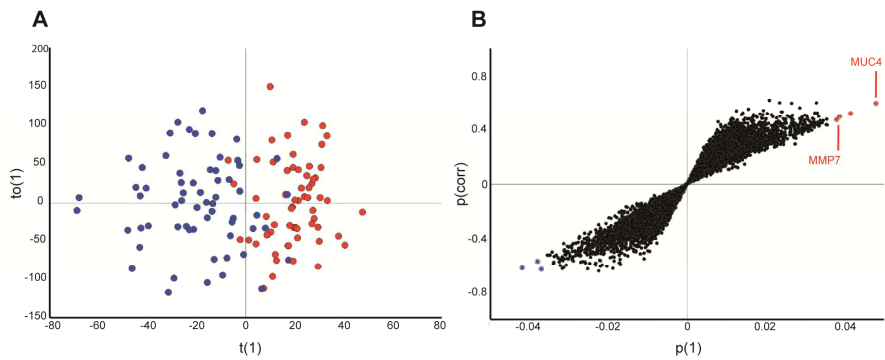
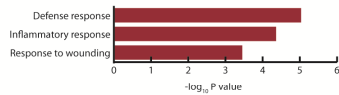


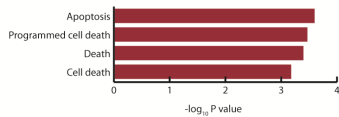
Figure 4.

**A**

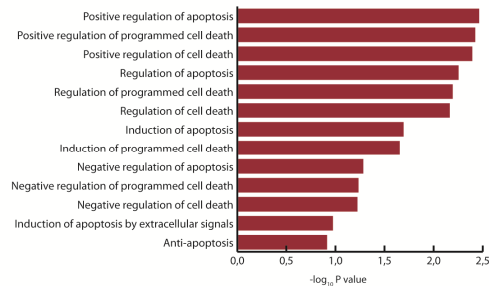
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Cluster 2 (EASE score 3.40)

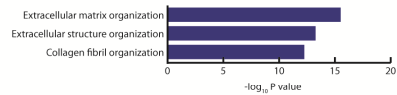


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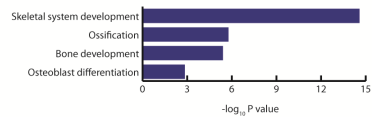


**B**

Cluster 1 (EASE score 13.64)



Cluster 2 (EASE score 7.13)



Cluster 3 (EASE score 5.02)

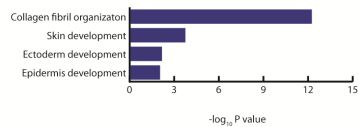


Figure 5.

